Culture-independent characterisation of microbial biofilm communities occluding biliary stents

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Summary

Biliary stents are medical implants placed in the bile duct to overcome obstructions. These artificial surfaces introduced into the human body are prone to colonisation by microorganisms, followed by biofilm formation and thus recurring obstruction of the bile duct. Previous studies on microbial colonisation of stents have only used culture-dependent approaches.

In this study, 133 biofilms from biliary stents were analysed for microbial community composition using culture-independent methods. Single-Strand-Conformation Polymorphism (SSCP) fingerprinting proved to be suitable to gain an overview of a high number of biofilm communities originating from different patients and hospitals. The use of SSCP was validated by comparison with results obtained by random sequencing of 16S rDNA clone libraries of selected microbial communities.

Overall, 62 bacterial phylotypes which cover 6 different bacterial phyla and represent a much broader diversity than those previously observed in culture-dependent studies were identified. The two most abundant phylotypes were *Veillonella* sp. and *Bifidobacterium* sp. occurring in more than 60% and 50% of all biofilm communities, respectively. Other abundant microorganisms were Streptococci, Enterococci, Fusobacteria, Enterobacteriaceae, Lactobacilli and *Bacteroides* species. In total, microorganisms similar to those constituting the duodenal microbiota were observed.

While influences on microbial composition due to hospital effects were minor and only substantiated by differences in the prevalence of Bifidobacteria, and differences in microbial community structure along the length of the stents were only visible by a higher prevalence of Lactobacilli at the stent end distal to the liver, a strong host-dependency was observed. This high variance and the significant similarity with human upper intestinal tract microbiota indicate a seemingly random colonisation of biliary stents depending on the prevailing duodenal microbiota of the patient at the time of stent placement. Furthermore, an increased similarity between stent communities from the same patient, particularly when stents were implanted simultaneously was evident.

However, colonisation was not entirely random. Community composition was dependent upon specific interactions between microorganisms resulting in a significant level of coaggregation by *Veillonella* and *Streptococcus*, and also shaped by the environmental conditions. On the one hand, species not abundant in the human gastrointestinal system were selected for and on the other hand, members of specific groups having adapted to survive in or to metabolise bile ingredients were abundant. In accordance, several enterococcal isolates were shown to exhibit bile salt hydrolase activity. However, bacteria capable of 7α-dehydroxylation of primary bile acids and taurine respiring microorganisms were evidently of minor importance.

Zusammenfassung

Gallengangstents sind medizinische Implantate, die in den Gallengang eingesetzt werden um Verengungen zu überbrücken. Diese in den Körper eingebrachten künstlichen Oberflächen sind für die Besiedelung durch Mikroorganismen, Biofilmbildung und eine daraus resultierende Blockierung des Gallengangs anfällig. Bisherige Studien zur mikrobiellen Besiedelung solcher Stents wurden nur mit kulturabhängigen Ansätze durchgeführt.

In dieser Arbeit wurden 133 Biofilme aus Gallengangstents auf die Zusammensetzung der mikrobiellen Gemeinschaft mit kulturunabhängigen Methoden untersucht. Single-Strand-Conformation Polymorphism (SSCP) fingerprinting erwies sich dabei als geeignete Methode um einen Gesamtüberblick über eine große Anzahl von Biofilmgemeinschaften von verschiedenen Patienten und Krankenhäusern zu gewinnen. Die Anwendung der SSCP Methode wurde durch einen Vergleich mit 16S rDNA Klonbanken ausgewählter mikrobieller Gemeinschaften validiert.

Insgesamt wurden 62 bakterielle Phylotypen identifiziert, die zu 6 verschiedenen Phyla gehören. Diese somit nachgewiesene Diversität ist weitaus höher als die bisher in kulturabhängigen Studien beobachtete. Die zwei häufigsten Phylotypen waren *Veillonella* sp. und *Bifidobacterium* sp., die in 60% und 50% aller Biofilmgemeinschaften auftraten. Andere häufig detektierte Mikroorganismen waren Streptokokken, Enterokokken, Fusobakterien, Enterobacteriaceae, Lactobazillen und Bacteroides Spezies. Insgesamt wurden Mikroorganismen nachgewiesen, die den in der duodenalen Gemeinschaft vorkommenden Mikroorganismen ähneln.

Der Einfluss der Krankenhäuser auf die mikrobielle Zusammensetzung war geringfügig und zeigte sich nur in Unterschieden in der Häufigkeit des Auftretens von Bifidobakterien. Unterschiede in der mikrobiellen Gemeinschaftsstruktur entlang der Länge der Stents bestanden nur in einer höheren Häufigkeit von Lactobazillen am distalen Leberende. Jedoch wurde eine starke Abhängigkeit der Zusammensetzung vom Wirt beobachtet. Sowohl die hohe Varianz als auch die signifikante Ähnlichkeit mit der mikrobiellen Gemeinschaft des menschlichen oberen Gastrointestinaltraktes deuten auf eine anscheinend zufällige Besiedlung der Gallengangstents hin, die von der vorherrschenden mikrobiellen Gemeinschaft des Patienten zum Zeitpunkt der Stentlegung abhängt. In Einklang damit wurde eine erhöhte Ähnlichkeit zwischen mikrobiellen Gemeinschaften des selben Patienten, vor allem wenn die Stents gleichzeitig implantiert waren, beobachtet.

Jedoch war die Besiedlung nicht vollkommen zufällig. Die Zusammensetzung der Gemeinschaft war sowohl von spezifischen Wechselwirkungen zwischen Mikroorganismen abhängig, die sich in einem signifikant erhöhten Niveau der Koaggregation von *Veillonella* und *Streptococcus* zeigten, als auch von den im Stent vorherrschenden Umweltbedingungen. Einerseits wurden Spezies selektiert, die nur in geringer Anzahl im menschlichen Gastrointestinaltrakt vorkommen, andererseits traten Vertreter spezifischer Gruppen gehäuft auf, die sich an das Überleben in

Galle oder den Metabolismus von Gallenbestandteilen angepasst haben. Dementsprechend zeigten mehrere hier isolierte Enterokokken eine Hydrolyse von Gallensalzen. Bakterien die eine 7α-Dehydroxylierung von primären Gallensäuren durchführen oder Taurin veratmen waren jedoch offensichtlich weniger bedeutend.

List of abbreviations

Amp	Ampicillin
ANOSIM	Analysis of similarity
APS	Ammonium persulfate
bai	Bile acid inducible
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BS	Surgery Clinic of Braunschweig
bsh	Bile salt hydrolase
℃	Degree Celsius
Conc.	Concentration
dATP	Deoxyadenosin-5'-triphosphate
dCTP	Deoxycytidin-5'-triphosphate
dGTP	Deoxyguanosin-5'-triphosphate
dTTP	Deoxythymidin-5'-triphosphate
dNTP	Mixture of dATP, dCTP, dGTP and dTTP
D	Simpson index
DGGE	Denaturing gradient gel electrophoresis
dist	Distilled
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	Exempli gratia (for example)
ERCP	Endoscopic retrograde cholangiopancreatography
et al.	Et alteri (and others)
FISH	Fluorescence in situ hybridisation
g	Gram
GI	Gastrointestinal
GIT	Gastrointestinal tract
H	Shannon index
h	Hour
Ig	Immunoglobulin
IPTG	Isopropyl-thio-ß-D-galactopyranoside
Kan	Kanamycin
kb	Kilo base pairs
l	Liter
M	Molar (mol/l)
m	Milli (10^{-3})
MDS	Multidimensional scaling
min	Minute
mM	Millimolar
µ	micro (10^{-6})
MW	Molecular weight
n	nano (10^{-9})
OD	Optical density

OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RDP II	Ribosomal Database Project II
rRNA	Ribosomal ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
SH	University Medical Center of Schleswig-Holstein in Kiel
SIMPER	Similarity percentages of species contributions
SSCP	Single-Strand-Conformation Polymorphism
ssDNA	Single stranded DNA
sec	Second
SZ	Medical Clinic of Salzgitter-Lebenstedt
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	N,N,N,N-Tetramethylethylenediamine
TGGE	Temperature gradient gel electrophoresis
™	Trademark
tpa	taurine:pyruvate aminotransferase
Tris	Tris(hydroxylmethyl)-aminomethane
U	Unit (enzyme unit)
UV	Ultraviolet light
v/v	Percent by volume
V	Volt
Vol	Volume
VS	Viridans Streptococci
x *g*	Multiples of acceleration of gravity
X-Gal	5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside

1 Introduction

1.1 Biliary stents

Malignancies of the pancreas and the heptobiliary system can result in obstruction of the bile duct and cause jaundice and cholangitis. Standard treatment as introduced by Soehendra is the endoscopic placement of a medical implant (Soehendra & Reynders-Frederix, 1980), a biliary stent that palliates the symptoms and furthermore improves the quality of life of patients (Ballinger *et al.*, 1994). As a consequence of the placement, however, the natural mechanical barrier between the intestine and the bile duct, the sphincter of oddi, is disrupted (see figure 1.1), and this can result in invasion of microorganisms via an ascending infection by duodenal biliary reflux (Sung *et al.*, 1992). The descending route of infection through the portal vein bloodstream (Sung *et al.*, 1991) could be another entry port for bacteria, the ascending route, though, being described as more probable in the current literature (Donelli *et al.*, 2007; Liu *et al.*, 1996; Sung, 1995), since an open connection between the colonised intestine and the biliary system is established. However, the descending route of infection was shown to be facilitated by a raised intrabiliary pressure (Sung *et al.*, 1991), and could thus play a role before insertion or in case of obstruction of stents. Subsequent attachment to the biliary stent surface can result in biofilm formation, stent occlusion and recurrent obstructive jaundice. Clogging of biliary stents, thus, is the major limitation of this therapy. Occlusion occurs after a mean of 4 to 6 months when large calibre plastic stents (10 to 11,5 French; 1 French corresponding to 0,33 mm) had been used (Gilbert *et al.*, 1992), and then stents have to be replaced. As further complication, stent lifetime decreases with repeated replacement, an effect thought to be due to an already existing microbial colonisation of the biliary system (Matsuda *et al.*, 1991), which is sterile under normal conditions (Csendes *et al.*, 1975; Scott, 1971).

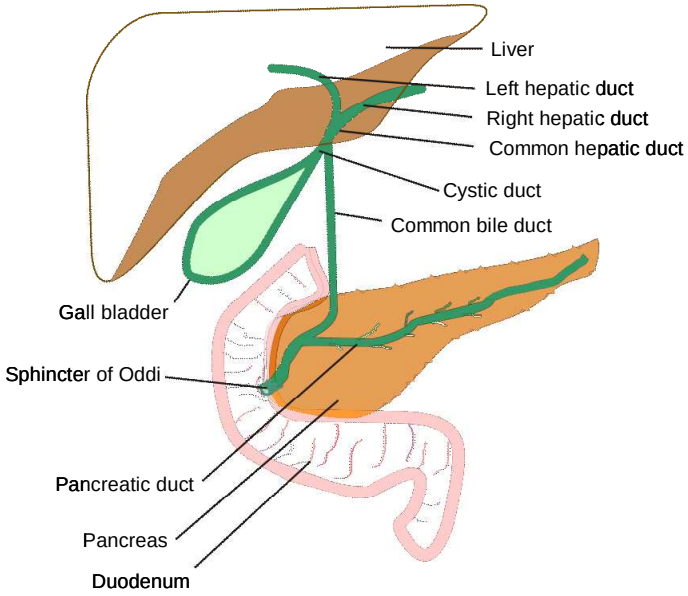

Figure 1.1: Overview of the biliary system. Picture modified after http://en.wikipedia.org/wiki/Image:Biliary_system.svg taken from Wikimedia Commens.

In order to prevent adhesion of microorganisms and prolong stent lifetime various approaches have been and are being currently tested, including prophylactic antibiotic treatment, variation of employed stents in design and material and coating of stent material with antimicrobial or hydrophilic agents (Jansen *et al.*, 1993; Leung *et al.*, 1992; Seitz *et al.*, 2007). Another approach is the placement of several stents at one time in the bile duct to increase the time until complete occlusion. Although the use of different materials and coatings appeared promising in vitro, efficiency in clinical trials was rarely observed (Catalano *et al.*, 2002; Schilling *et al.*, 2003; van Berkel *et al.*, 2004). The only strategy that seems efficient in prolonging stent patency is increase of the diameter (Faigel, 2000; Siegel *et al.*, 1988; Speer *et al.*, 1988a) although there are some studies that show no clear effect (Kadakia & Starnes, 1992; Pereira-Lima *et al.*, 1996), thus suggesting the advantage of larger diameters to be marginal. The size of plastic biliary stents is limited to 12 French due to the size of the available duodenoscopes, through which the stents are introduced during the endoscopic placement. Another approach to reach higher inner diameters is the use of self-expanding metal stents, which generally show a longer patency but cannot be removed, may favour ingrowth of tumour and epithelial cells and are more expensive (Donelli *et al.*, 2007). The most recent approach to prevent biliary stent occlusion is the development of antireflux biliary stents, which allow only one-directional flow of bile (Reddy *et al.*, 2006).

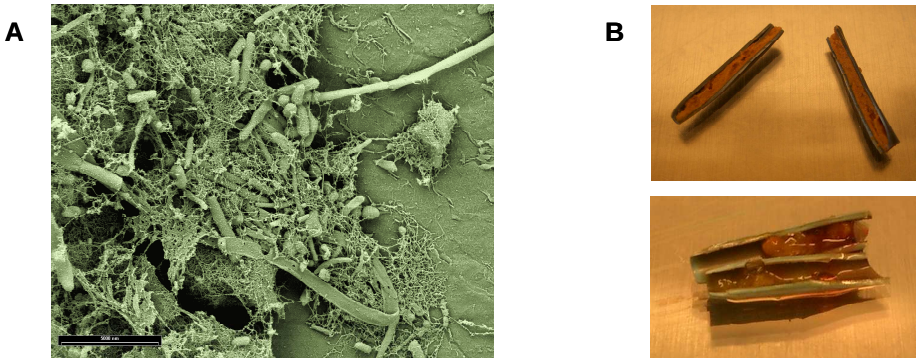

Figure 1.2: Scanning electron micrograph of a biliary stent biofilm (A) and longitudinal sectioning through two occluded biliary stents (B).

The biofilms, which establish in biliary stents are composed of mixed microbial communities and embedded in a matrix of amorphous material (see figure 1.2 A and B). Microorganisms, mucoproteins, cholesterol monohydrate crystals, calcium bilirubinate and other calcium salts form a sludge that supports clogging of the stent (Leung *et al.*, 1988). The composition of biliary stent microbial communities was up to now mainly characterised using typical methods of clinical microbiology, which are based on culturing of microorganisms. A comprehensive summary of microorganisms isolated from biliary stent biofilms as described in the literature is given in table 1.1. Predominantly facultative and strict anaerobic bacteria and some fungi (mainly *Candida* sp.) were isolated, with *Enterococcus* sp. being the most abundant gram-positive and *Escherichia coli* and *Klebsiella* sp. being the most abundant gram-negative organisms among the facultative anaerobes. *Clostridium* sp. was the most prominent strict anaerobic species. However, variations in the ratio between facultative and strict anaerobic bacteria as well as in the species composition were evident between the different studies, which is, among other factors, probably due to differences in the applied cultivation media and techniques. Specifically, in the study of Leung et al. mainly strict anaerobic bacteria besides gram-positive facultative anaerobes and fungi, but no gram-negative facultative anaerobes were isolated. The authors speculate that strict anaerobic bacteria play an important role in early attachment, since all stents have been removed after a mean of 33 days. However, all patients received antibiotic treatment targeted against aerobic (facultative anaerobic) gram-negative bacteria immediately before or after stent placement. In general, however, a similar composition of microbial communities was observed in the different studies.

Table 1.1: Microorganisms isolated from biliary stent biofilms and their abundance as reported in different studies.

	(Speer *et al.*, 1988b) (n=31)*	(Dowidar *et al.*, 1991) (n=26)*	(Molinari *et al.*, 1996) (n=25)*	(Di Rosa *et al.*, 1999) (n=30)*	(Leung *et al.*, 2000b) (n=18)*	(Zhang *et al.*, 2003) (n=8)*
Facultative anaerobic bacteria						
Gram-positive						
Bacillus sp.				1	3	
Enterococcus sp.	3	7	19	24	8	4
Pediococcus sp.				1		
Streptococcus sp.	6	3		1		2
Staphylococcus sp.	8	1		6	2	2
Gram-negative						
Aeromonas hydrophila	1					
Citrobacter sp.	10		10	1		2
Escherichia coli	28	12	13	4		3
Enterobacter sp.	6	5	12	2		3
Hafnia alvei	3	2				
Klebsiella sp.	12	13	9	3		2
Morganella morganii	1	1	5			
Pantoea sp.			3			
Proteus sp.	5	2	1			
Providencia rettgeri			1			
Pseudomonas sp.	1	5	5	1		1
Serratia sp.						2
Yersinia enterocolitica	1					
Strict anaerobic bacteria						
Gram-positive						
Clostridium sp.	1	3			17	
Gram-negative						
Bacteroides sp.		1			2	
Fusobacterium sp.		1				
Veillonella sp.				1		
Fungi						
Aspergillus sp.					2	
Candida sp.	1	4	3	5	1	

* n = Number of stents subjected to isolation.

The only study on biliary stent microbial community structure that is based on culture-independent methods (Swidsinski *et al.*, 2005) used fluorescence in situ hybridisation (FISH) with mainly group specific probes e.g. targeted against Alpha-, Beta- and Gamma- Proteobacteria, but also some genus specific probes e.g. targeted against Streptococci or Bifidobacteria. The analysis of fifty-two biliary stents revealed the presence of a rich, complex bacterial community consisting mainly of *Bacteroides* sp., Gamma-Proteobacteria, Firmicutes and members of the *Clostridium lituseburense* group embedded in the sludge of narrowed and partially occluded stents. However,

whereas hybridisation signals were clearly visible for 10 to 20 seconds in narrowed stents, the analysis of partially and fully occluded stents required very intense conditions, like hybridisation for over 12 hours and signals extinguished rapidly after excitation. The number of bacteria detectable by this approach decreased strongly with increasing degree of occlusion. This is most probably due to poor hybridisation, since a large number of structures resembling bacteria were observed through autofluorescence and gram staining in completely occluded stents. Furthermore, the authors analysed biopsies of the duodenum, bile ducts and gall bladders of patients that did not have a biliary stent. The 21 examined duodenal biopsies, 5 bile ducts and 20 gall bladders did not show any biofilm on the surface, indicating sterile bile ducts when the sphincter separating the bile ducts from the duodenum is not interrupted by a stent, and no permanently attached microbiota in the mucosa of the duodenum. However, a transient microbiota seems to be present in the duodenum, since several studies were able to detect microorganisms from duodenal samples as discussed below.

1.2 Microbiota inherent to the gastrointestinal system

The intestine is the most heavily colonised part of the human body, where total microbial cells outnumber at least ten times the cell number of a human being (Bäckhed *et al.*, 2005). The diversity of bacteria that has been observed in the human gut is displayed in figure 1.3. The diversity at the division level is low in comparison to e.g. soil, which is inhabited by members of at least 20 bacterial divisions (Dunbar *et al.*, 2002). In the oral cavity and the oesophagus the bacterial division TM7 is detected additionally to the ones observed in the human gut.

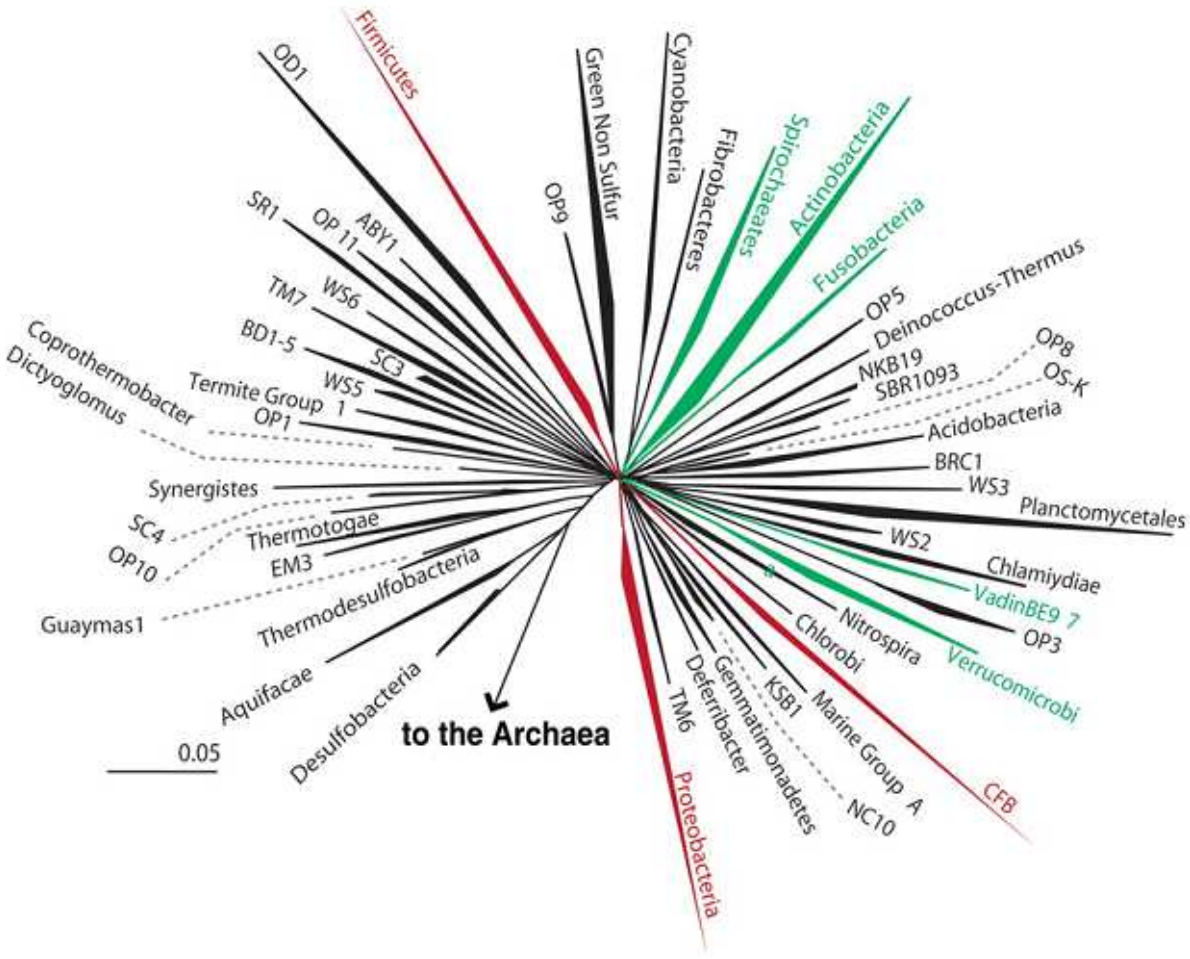

Figure 1.3: Phylogenetic tree of the domain bacteria based on 8903 representative 16*S* rRNA gene sequences as presented by Bäckhed *et al.* (Bäckhed *et al.*, 2005). Wedges represent divisions: Those numerically abundant in the human gut are indicated in red, divisions rarely found are indicated in green, and those probably absent from the gut are indicated in black. Wedge length is a measure of evolutionary distance from the common ancestor.

However, despite the presence of members of only a few bacterial divisions, the intestinal communities are highly diverse and contain hundreds of bacterial species (Dethlefsen *et al.*, 2006). The gut microbiome, the collective genome of the gut microbiota, is estimated to contain 100 times the number of genes of the human genome (Bäckhed *et al.*, 2005) and can be regarded as a microbial organ fulfilling important protective, structural and metabolic functions (O'Hara & Shanahan, 2006). However, the whole gastrointestinal system (figure 1.4) consists of quite diverse habitats for microbial communities, which change in composition and abundances along its course.

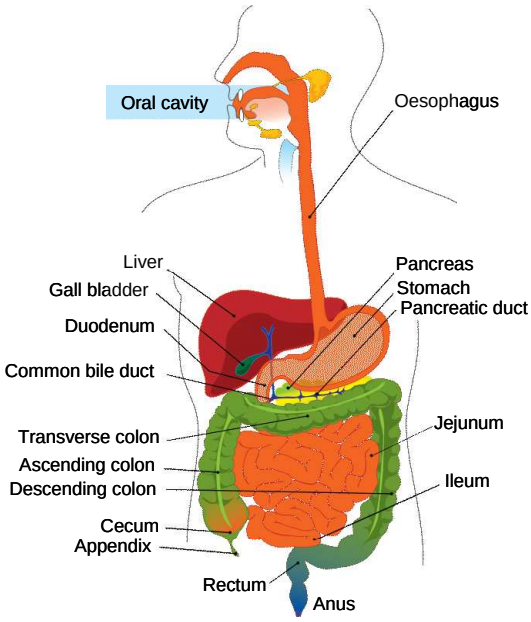

Figure 1.4: Overview of the gastrointestinal system. Picture modified after http://en.wikipedia.org/wiki/Image:Digestive_system_diagram.svg taken from Wikimedia Commens.

The duodenum, which is the most likely source for microorganisms invading the biliary system after placement of a biliary stent, is characterised by rapid peristalsis and a short transit time for the lumenal contents. Large quantities of fluids (drinks, saliva, gastric juices, bile, pancreatic juice and intestinal fluids) are guided through the small intestine and the pH at the level of the duodenum ranges from 5.7 to 6.4 (Wilson, 2005). The composition of the duodenal microbiota is until now only determined using a culture-dependent approach. Two studies from 1989 (Bernhardt & Knoke, 1989; Skar *et al.*, 1989) used an endoscopic sampling approach, which may introduce

contamination to samples originating from the microbiota of the oral cavity, where the endoscope is passed during the sampling procedure. Bernhardt (Bernhardt & Knoke, 1989) observed microbial growth from more than 90% of all investigated samples obtained from 400 patients. The observed microbiota were classified into colonisation types according to the types of isolated microorganisms. The “normal” colonisation type comprised mainly Streptococci, Lactobacilli and various anaerobic cocci. Moreover, Enterobacteriaceae, Staphylococci, *Bifidobacterium* sp., *Bacteroides* sp., *Fusobacterium* sp. and yeast were observed in low abundances. The total cell number was estimated as $\leq 10^3$ cfu/ml of total aerobes, and $\leq 10^4$ cfu/ml of total anaerobes. The other colonisation types were mainly characterised by elevated cell counts of one or several types of microorganisms (up to 10^6 cfu/ml of total aerobes and 5×10^6 cfu/ml of total anaerobes) or the presence of additional microorganisms like Enterococci. However, the time of sampling in relation to nutrient uptake was proposed to have a strong effect on viable cell counts, which were elevated after entry of chyme from the stomach and returned to baseline values of $\leq 10^3$ cfu/ml within one hour (Wilson, 2005). Similar results were obtained by Skar *et al.* (Skar *et al.*, 1989) who, by analysing samples from 25 individuals indicated the presence of duodenal microbiota consisting mainly of gram-positive bacteria with α-haemolytic Streptococci being the most abundant. Furthermore, Lactobacilli, Enterococci, other Streptococci and Staphylococci were observed as well as some strict anaerobes (*Clostridium* sp. and *Bacteroides* sp.*)* and fungi (*Candida* sp.). An additional study is based on samples obtained during surgery of patients with abdominal trauma and gastric or gastrointestinal disorders, thus being less prone to contamination during sampling (Thadepalli *et al.*, 1979). Culturing was only possible in 17% of 36 investigated patients and strict anaerobes were not observed in any case. Microorganisms detected were *Enterococcus faecalis*, *Klebsiella* sp., *Staphylococcus aureus*, *Lactobacillus* sp. and *Escherichia coli*. Similarly, the authors were able to obtain isolates only from 29% and 44% of samples from the jejunum and the ileum, respectively, which were accordingly claimed to contain a scarce microbiota, an assumption clearly contrasted by recent culture-independent analyses (Hayashi *et al.*, 2005; Wang *et al.*, 2005). Evidently, the low amount and diversity of the duodenal microbiota in comparison to the two above mentioned studies are due to the limited culturing conditions applied and thus underline the restricted usefulness of culturing studies. Nevertheless, culture-independent studies indicated the duodenal microbiota to be dominated by acid-tolerant species like Lactobacilli and

Streptococci, however, a wide variety of other microorganisms may also be present depending on the host and time of sampling.

Recently, numerous studies using culture-independent methods were carried out to explore the microbiota of the gastrointestinal system, several of these based on random sequencing of 16S rDNA clone libraries (Bik *et al.*, 2006; Eckburg *et al.*, 2005; Pei *et al.*, 2004). The spatially most closest to the duodenum are two studies investigating mucosal biopsies (Wang *et al.*, 2005) and gut contents (Hayashi *et al.*, 2005) of the human jejunum, respectively, in comparison with the ileum and different sections of the colon. Wang *et al.* analysed samples of only one healthy individual and proved members of the genus *Streptococcus* to be dominant (68% of all clones) in the jejunum of the host. Moreover, Proteobacteria (mainly gamma-Proteobacteria), Clostridiae (cluster IX and XI), Bacteroidetes (all *Prevotella* sp.), Fusobacteria (*Fusobacterium* sp.) and Actinobacteria (*Micrococcus* sp.) were observed. The study by Hayashi *et al.* included three subjects and the three resulting clone jejunal libraries were dominated by *Lactobacillus* sp. *Streptococcus* sp., *Enterobacter* sp. and *Klebsiella* sp., respectively. Major inter-individual differences were apparent in this study, although this could also be due to the characterisation of the transient gut contents, which presumably change more rapidly in contrast to the microbiota attached to the mucosa. Both studies observed the microbiota of the jejunum to be the least diverse among all investigated habitats (jejunum, ileum, caecum, colon and rectum), mainly comprising facultative anaerobes and being considerably different to the microbiota of the large intestine, which are characterised by a high number of fastidious anaerobes and yet uncultivated and uncharacterised bacterial species. This is in accordance with the proposed proximal-to-distal gradient of the gastrointestinal system from ileum to distal colon in which the number of phylogenetic groups increases and shared phylogenetic groups are least between the most distant sites (Wang *et al.*, 2003).

In contrast to the jejunum and ileum, which are colonised by 10^{4-7} cfu/ml of bacteria, the microbiota of the colon is characterised by higher population numbers, increasing from approximately 10^{8} cfu/ml in the caecum to approximately 10^{11-12} cfu/ml in the faeces (Marteau *et al.*, 2001; O'Hara & Shanahan, 2006). The number of species is estimated to be approximately 800 (Bäckhed *et al.*, 2005) affiliated with nine bacterial phyla and one archeal phylum (Ley *et al.*, 2006). A comprehensive culture-independent

study of mucosal samples of six subdivisions of the colon and faecal samples of three healthy subjects (Eckburg *et al.*, 2005) showed the microbiota to be dominated by the two phyla of Firmicutes and Bacteroidetes, with a majority of the sequences observed closely related to uncultivated species or novel microorganisms. Similar to the studies on the small intestinal microbiota of several individuals, significant intersubject differences were observed, accounting for the greatest amount of variability within the data. Most of the remaining variability could be explained by differences between mucosal and faecal communities within individuals, whereas among mucosal libraries from the same subjects little variation was observed. The proportion of obligate anaerobes was strongly elevated compared to the small intestine and further increases along the large intestine, with about 25% of the microbial community of the caecum but only 1% of the respective community of the faeces being represented by facultative anaerobes (Marteau *et al.*, 2001).

Also the microbiota of the oral cavity, the oesophagus and the stomach have been recently studied using molecular techniques. In a study of nine sites within the oral cavity of five healthy subjects (Aas *et al.*, 2005) over 700 bacterial phylotypes representing six different phyla (Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteira and TM7) were observed. More than half of the detected bacterial phylotypes have not yet been cultured. Phylotypes common to all sites were affiliated with the genera *Gemella, Granulicatella, Streptococcus* and *Veillonella*, however different sites within the oral cavity were found to harbour distinct multispecies communities. The surface attachment properties of the microorganisms seem to be an important selective feature for the establishment of these communities. A study of mucosal samples from the oesophagus of four healthy subjects (Pei *et al.*, 2004) observed 95 bacterial phylotypes representing the same six phyla as found by Aas *et al.* (Aas *et al.*, 2005) in the oral cavity. The major distinction to the oral cavity microbiota, however, was the high abundance of known and cultivable microorganisms. Thus, the oesophagus seems to harbour similar but less complex microbiota compared to the oral cavity on its mucosal surfaces. The stomach in contrast to the oral cavity was traditionally regarded as inhospitable environment for microorganisms mainly due to the gastric acidity. However, *Helicobacter pylori* is regarded as a frequent coloniser of the stomach today (Bik *et al.*, 2006). Furthermore, a molecular study of the human gastric mucosa of 23 individuals (Bik *et al.*, 2006) observed 128 bacterial phylotypes representing eight phyla. Gastric bacterial communities were dominated by

microorganisms affiliated with the five phyla of Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria. Fifty percent of the phylotypes were affiliated with yet uncultivated bacteria and about 70% have been previously described as oral cavity microbiota. The higher-order taxon diversity, however, was found to be similar to the one described for the lower gastrointestinal tract. The authors pointed out that the detected bacterial DNA sequences may represent transiently ingested bacteria and not necessarily a resident microbiota.

1.3 Molecular methods for microbial community analysis and use of 16S ribosomal RNA genes as phylogenetic markers

The use of molecular methods in microbial ecology has strongly increased the capability to describe the structure of microbial communities. It has revealed that the diversity of the microbial world was heavily underestimated by the use of culturing methods (Hugenholtz *et al.*, 1998). Now the challenge to microbiologists is to culture representatives of newly detected phyla in order to gain insight into their physiology and environmental role. The comparative sequence analysis using small ribosomal RNAs (16S and 18S rRNA) as phylogenetic marker molecules was introduced by Woese and colleagues who as well proposed the three domains of life (Eucarya, Archaea and Bacteria) (Woese & Fox, 1977). The 16S rRNA, or in most cases more precisely its counterpart the 16S rDNA, is the most widely used phylogenetic bacterial marker molecule. The most recent publication of the Ribosomal Database Project II (Cole *et al.*, 2007) quotes about 260.000 entries in the release 9.42 of September 2006, whereas the current release 9.52 (July 2007) already contains more than 390.000 entries. The 16S rRNA is an ideal phylogenetic marker molecule due to its central role in the translation of nucleic acids, which makes it indispensable to living organisms, it is ubiquitous and has a mosaic like structure of conserved and variable regions. Furthermore, it has a high amount of informative positions (approximately 1500 nt) outnumbered only by the 23S rRNA (approximately 2900 nt) (Ludwig *et al.*, 1998).

However, PCR-based analysis of microbial communities has as well its own specific limitations and error sources like differential PCR amplification of different rDNA templates, formation of PCR artefacts or sequence variations due to ribosomal RNA

operon heterogeneity (von Wintzingerode *et al.*, 1997), nevertheless if precautions are taken and sequence data are analysed carefully, the increase in information which is gained is impressive. Thus, in the current study two molecular methods for analysis of microbial community structures, namely Single-Strand-Conformation Polymorphism (SSCP) fingerprinting and random sequencing of 16S rDNA clone libraries, were applied concurrently and compared to estimate the reliability of microbial community analysis.

In general fingerprinting methods are based on the amplification of a marker gene from total community DNA, representing a mixture of genomic DNA of all microorganisms present. The amplified fragments are then separated, resulting in fingerprints representative for the original community composition, allowing thus the comparison of different communities or monitoring of shifts in community composition. The most common fingerprinting methods separate amplicons according to their sequence specific melting behaviour (Denaturing Gradient Gel Electrophoresis - DGGE, Temperature Gradient Gel Electrophoresis – TGGE) (Muyzer, 1999), their secondary structure of single-stranded DNA (SSCP) (Schwieger & Tebbe, 1998) or their length after digestion with sequence specific restriction enzymes (terminal restriction fragment length polymorphism - T-RFLP) (Marsh, 1999). The resolution of fingerprinting methods in general is limited to the predominant microorganisms in the communities, however, besides universal eubacterial primers targeting the 16S rDNA also the application of group-specific primers targeting the 16S rDNA of predefined phylogenetic groups of interest (Salles *et al.*, 2002) or primers against functional genes (Junca & Pieper, 2004; Nicolaisen & Ramsing, 2002) is possible, thus increasing the resolution for a specific bacterial phylogenetic or functional group. Fingerprinting methods allow for a rapid assessment of microbial community structures, thus enabling screening of high sample numbers in a relatively short amount of time. The large data-sets which are generated using fingerprinting methods can be analysed by multivariate statistical methods to reveal similarities and dissimilarities between microbial communities as analysis by eye becomes impossible (Nelson, 2007; Van der Gucht *et al.*, 2005).

SSCP fingerprinting was originally developed as a method to detect point mutations in clinical research (Orita *et al.*, 1989) and later optimised for the analysis of microbial communities (Schwieger & Tebbe, 1998). For community analysis in the current study universal eubacterial primers were applied to amplify a fragment of the 16S rRNA

genes present in the communities, with the reverse primer being phosphorylated at the 5' end. Using lambda exonuclease, which specifically recognises the phosphorylated DNA strand, the obtained amplicons are digested to single-stranded DNA. The single strands acquire a sequence dependent spatial conformation through boiling and fast chilling on ice and can be separated accordingly on nondenaturing polyacrylamide gels. The resulting band patterns represent microorganisms present in the analysed communities. Additionally, predominant microorganisms can be phylogenetically classified through reamplification and sequencing of the major bands of fingerprints. SSCP fingerprinting has been successfully applied to different habitats, using both 16S rRNA and functional genes for separation (Eichler *et al.*, 2006; Fracchia *et al.*, 2006; Junca & Pieper, 2004; Witzig *et al.*, 2006). The amplified fragments that are generated using the universal eubacterial primers applied in this study comprise the variable regions V4 and V5 of the 16S rRNA (Neefs *et al.*, 1993), which are highly informative regions and have been shown to be appropriate for SSCP fingerprinting (Schmalenberger *et al.*, 2001). However, the information retrieved is restricted to about 25% of the informative positions of the whole 16S rRNA molecule, and thus, discriminatory power is slightly smaller compared to the analysis of full length 16S rDNA segments. In contrast to SSCP fingerprinting random sequencing of 16S rDNA clone libraries acquires information over the nearly complete length of the 16S rDNA molecule. Thus, a more detailed picture of phylotypes present in a community is generated. However, the method is too time-consuming and costly to be applicable to a high number of samples. Thus, the analysis of 16S rDNA clone libraries was used to elucidate the fine scale structure of selected microbial communities and to validate the use of SSCP fingerprinting for analysis of biliary stent microbial communities. However, even analysis of full length 16S rDNA sequences may in some cases, not be sufficiently discriminatory to permit resolution of intrageneric relationships. To obtain better separation of bacterial species other molecular markers, e.g. RNA polymerase delta 70 factor (rpoD), ATPase subunits or DNA gyrase (Yamamoto & Harayama, 1998) have been used. Functional proteins are characterised by a higher diversity compared to ribosomal RNAs, thus yielding a finer resolution in comparative sequence analysis. As an example, 16S rRNA gene sequences are useful for describing phylogenetic relationships between distantly related Enterobacteriaceae, whereas DNA gyrase gyrB sequence comparison were found to be useful for inferring intra- and some intergeneric relationships (Dauga, 2002).

1.4 Metabolic activities present in biliary stent biofilms?

In addition to the question which microorganisms are present in biliary stent biofilms, the succeeding question is what metabolic transformations and reactions may take place in this habitat and favour growth of specific groups of microorganisms. Table 1.2 shows the composition of human liver and cystic bile, with cystic bile being the concentrated form of liver bile as stored in the gallbladder. Bile is a medium with moderate pH and inhibitory concentration of bile salts. The main mode of antimicrobial action of bile salts is their damaging effect on cell membranes, as shown in experiments using erythrocytes (De Boever *et al.*, 2000) and indicated by the majority of bile-sensitive mutants being affected in genes associated with the maintainance of membrane integrity (Begley *et al.*, 2005a). The inhibitory effects increase with concentration and hydrophobicity of bile salts. However, the inhibitory effect may be attenuated *in vivo,* as bile salts are present in mixed micellar solutions reducing significantly the bacteriostatic properties (Sung *et al.*, 1993).

Table 1.2: Composition of human liver and cystic bile (Geigy, 1968a).

	Liver bile (A-bile)	Cystic bile (B-bile)
Appearance	Golden, yellowish orange	Brownish black, brownish green
Amount per day	250-1100 ml	-
Specific gravity	0.995-1.008	1.008-1.034
pH	6.2-8.5	5.6-8.0
Water	97-98%	84%
Bile salts	6.5-14 g/l	115 g/l
Cholesterol	0.8-1.8 g/l	3.1-16.2 g/l
Phospholipids	1.0-4.3 g/l	15-53 g/l
Bilirubin	0.65 g/l	2.94 g/l
Protein	1.8 g/l	4.5 g/l
Fatty acids	1.6-4.1 g/l	24 g/l
Total carbohydrates	0.35-0.91 g/l	2.4 g/l
Urea	236 mg/l	200-450 mg/l
Mucins	-	1-4%

The major constituent of bile are bile salts (see table 1.2). The corresponding acids are produced from cholesterol in a multienzyme process and conjugated to either glycine or taurine in human liver cells (Kevresan *et al.*, 2006) (figure 1.5). Bile formed in the

liver is secreted into small channels, the canaliculi, which merge into the hepatic ducts. Liver bile is then stored in a concentrated form in the gallbladder (see table 1.2) from where it is released upon nutrition uptake, by stimulation of gallbladder contraction through hormones. Released to the small intestine conjugated bile acids act as biological detergent and assist in lipid emulsification, digestion and absorption. Reabsorbtion of conjugated and unconjugated bile acids takes place by passive diffusion along the entire gut and active transport in the distal ileum. After entering the portal bloodstream bile acids are taken up by hepatocytes, reconjugated and resecreted into bile, in a process called enterohepatic circulation.

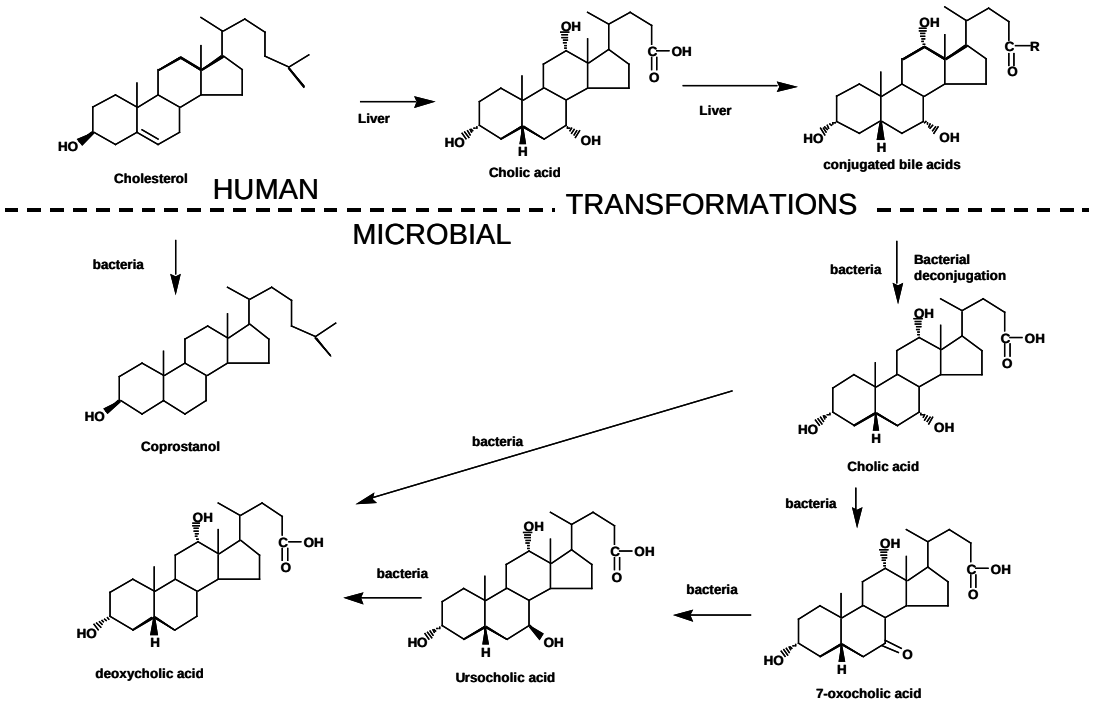

Figure 1.5: Cholesterol and bile acid metabolism, including transformations by human and microbial enzymes.

Bacterial bile acid modifications are known to occur in the large bowel. The three main reactions are deconjugation, 7α-dehydroxylation and 7α-dehydrogenation (Begley *et al.*, 2005a) (figure 1.5). Deconjugation through hydrolysis has to occur before other modifications are possible and leads to the cleavage of glycine or taurine, releasing free bile acids. Bile salt hydrolase (bsh) activity is expressed by a large number of mostly gram-positive intestinal bacteria (Begley *et al.*, 2006). The gene was cloned from several microorganisms like *Enterococcus faecium*, *Bifidobacterium adolescentis*,

Lactobacillus plantarum, Clostridium perfringens and *Bacteroides vulgatus* (Christiaens *et al.*, 1992; Coleman & Hudson, 1995; Kawamoto *et al.*, 1989; Kim *et al.*, 2005; Wijaya *et al.*, 2004) and is widespread among strains of these genera associated with the gastrointestinal environment (Begley *et al.*, 2006). The gastrointestinal pathogen *Listeria monocytogenes* as well possesses a gene coding for a bsh (Dussurget *et al.*, 2002). The reasons for bacterial bsh activity are not well understood, although several hypotheses are in discussion (Begley *et al.*, 2006). A nutritional advantage can arise from the released amino acids, which could potentially be used as carbon, nitrogen or energy sources, whereas the steroid backbone of bile salts is generally not used for energy metabolism under anaerobic conditions. However, Harder described the mineralisation of cholesterol by a novel denitrifying bacterium under strictly anoxic conditions (Harder & Probian, 1997) proving that the steroid backbone can be cleaved in the absence of oxygen. Another possible advantage of bsh activity is the alteration of bacterial membrane characteristics. It has been proposed that bsh activity favours the incorporation of cholesterol and bile acids into the membranes, hereby conferring protection against perturbations of the structure and integrity of bacterial membranes (Dambekodi & Gilliland, 1998; Taranto *et al.*, 2003). One further advantage may be the increase of gastrointestinal survival and persistence since bsh activity may combat the deleterious effects of bile. Accordingly, *Listeria monocytogenes* faecal carriage was reduced after oral infection of guinea pigs with a bsh mutant and reduced virulence and liver colonisation were observed in intravenous inoculated mice (Dussurget *et al.*, 2002). Finally, a bile detoxification mechanism could be the driving force for bsh activity and it was reported that mutations in *bsh* renders bacterial cells significantly more sensitive to bile and bile acids (Begley *et al.*, 2005b; De Smet *et al.*, 1995; Dussurget *et al.*, 2002; Grill *et al.*, 2000). Although the mechanism by which bsh activity plays a role in tolerance to bile is not fully understood, it has been proposed that the nondissociated bile salts may exhibit toxicity through intracellular acidification. Bsh activity forming the weaker unconjugated bile acids may lower this toxic effect (De Smet *et al.*, 1995). Additionally, deconjugation of bile acids may be coupled to a further detoxification reaction of the formed unconjugated bile acids. The 7α-dehydroxylation encoded by bile acid inducible (*bai*) genes of Clostridiales could be such a coupled reaction. Primary bile acids (cholic acid and chenodeoxycholic acid) are converted in a multistep process to secondary bile acids (deoxycholic acid and lithocholic acid), which precipitate at a moderately acidic pH, thus restraining their toxicity (De Boever & Verstraete, 1999). An alternative reaction is the 7α-dehydrogenation of primary bile

acids leading to the formation of 7-oxocholic acid and 7-oxolithocholic acid, which can be further epimerised to ursocholic acid and ursodeoxycholic acid (Bortolini *et al.*, 1997). The latter is used as a pharmaceutical drug to facilitate bile flow. These bacterial modifications are all known to occur in the large bowel (Ridlon *et al.*, 2006). Since the substrates are also present in the bile ducts and accordingly in biliary stents, it would be interesting to know whether such transformations occur also in biliary stent biofilm communities.

1.5 Possible mechanisms contributing to biliary stent occlusion

Several factors and processes are assumed to contribute to the event of clogging of a biliary stent. The deposition of host proteins is a well known effect after placement of indwelling medical devices in the human body and has been shown for e.g. central venous catheters or contact lenses (Gudmundsson *et al.*, 1985; Raad, 1998). A protein layer could also be shown to have formed in a flow cell model after perfusion for 24h with human bile (Yu *et al.*, 1996). The main proteins absorbed on biliary stent surfaces of stents removed from patients were identified as fibronectin and vitronectin (Yu *et al.*, 1996). Many gram-positive bacteria possess specific adhesins which bind to extracellular matrix proteins of the host and several fibronectin binding proteins involved in adherence and entry into host cells have been reported for Streptococci and Staphylococci (Joh *et al.*, 1999; Schwarz-Linek *et al.*, 2004). It can thus be assumed that the initial attachment of bacteria to biliary stent surfaces is facilitated by those host proteins absorbed to the stent surface. Several studies actually observed a preconditioning effect of protein coated stents *in vitro*, thus facilitating the absorption of bacteria (Yu *et al.*, 1996; Zhang *et al.*, 2002).

Microorganisms attaching to the newly introduced surface clearly play a major role in the event of clogging. In an *in vitro* model, stents perfused with bile infected with biliary stent isolates accumulated significantly more sludge than those perfused with sterile bile, and of seven different species tested, specifically perfusion with *Staphylococcus aureus* and *Pseudomonas aeruginosa* resulted in significant amounts of material attached to the inner stent walls (Dowidar *et al.*, 1992). The biofilms forming in biliary stents are characterised by mixed microbial communities, and a synergistic effect between gram-negative and gram-positive bacteria was observed *in vitro* when

bacterial suspensions of *Escherichia coli* and *Enterococccus* sp. were perfused through fragments of biliary stents. Whereas *E. coli* adhered rapidly and in high numbers to the stent surface, *Enterococcus* sp. alone did not form a stable biofilm. However, after preconditioning of the surface by perfusion with *E. coli*, *Enterococcus* sp. showed an increased adherence (Leung *et al.*, 1998). Thus, biofilm formation is enhanced through the cooperation of different types of bacteria. After initial attachment and premature biofilm formation, a kind of sludge is accumulated that finally leads to stent occlusion. A chemical analysis of the deposited material in biliary stents identified proteins, bilirubin, calcium palmitate, calcium bilirubinate and cholesterol as main components (Costa *et al.*, 2001). While precipitation of cholesterol occurs after oversaturation of cholesterol in phospholipid vesicles in which it is solubilised (Donelli *et al.*, 2007), calcium bilirubinate and calcium palmitate precipitates are thought to be formed through the action of bacterial enzymes (Liu *et al.*, 1996; Sung, 1995). Bilirubin is conjugated to glucuronic acid in the liver to render it water soluble and is excreted to bile in its diglucuronide form, however, minor amounts of the monoglucuronide or the unconjugated form are also observed (Wu *et al.*, 1980). Beta-glucuronidase, an enzyme known to be harboured by a wide range of gram-negative and gram-positive bacteria is capable of deconjugation of bilirubin diglucuronide resulting in the formation bilirubin acid which, in the presence of calcium precipitates as calcium bilirubinate (Leung *et al.*, 2001). The activity of this enzyme was tested in approximately 200 bacterial biliary stents isolates pre-grown in human bile. A significant portion of 60 isolates, including *Escherichia coli*, *Clostridium perfringens*, *Klebsiella* sp., *Enterococcus* sp. and *Streptococcus* sp. strains, showed a bilirubin diglucuronide deconjugating activity, with the activity being highest in *Clostridium perfringens* isolates (Leung *et al.*, 2001), suggesting this activity to contribute to biliary stent clogging. Another enzymatic reaction which may favour sludge accumulation is bacterial phospholipase C, an enzyme capable of releasing palmitic acid from phospholipids, a process which can be followed by precipitation of calcium palmitate. Phospholipase C activity was observed in all isolated 17 *Clostridium* sp. from biliary stents, while this enzymatic activity was absent in all other 18 bacterial isolates comprising strains of *Enterococcus* sp., *Bacillus* sp., *Staphylococcus* sp. and *Bacteroides fragilis*. Thus, the authors speculate that *Clostridium* sp. may play an important role in sludge accumulation. However, facultative anaerobic gram-negative micororganisms have not been isolated in that study (Leung *et al.*, 2000b) and were thus not assessed.

Additional information on the composition of sludge resulting in stent clogging was obtained by electron and confocal microscopy or studies applying histological staining procedures (Chan *et al.*, 1998; van Berkel *et al.*, 2005; Weickert *et al.*, 2001; Zhang *et al.*, 2003). Mucin, a high molecular weight glycoprotein which is secreted by the gallbladder epithelium and the primary constituent of gallbladder mucus, was identified as constituent of biliary sludge (Zhang *et al.*, 2003). The presence of mucin was shown along the inner wall of the stents as well as on the surface of insoluble deposits and bacterial microcolonies in all of about 30 biliary stents analysed. By acting as kind of cement among deposited substances and clumps of bacteria it may contribute significantly to stent occlusion (Zhang *et al.*, 2003).

Another important factor seems to be the entry of dietary fibres into the stents through duodenal reflux. In 38 of 100 stents analysed macroscopically and cytologically plant material was observed, whereas ingrowth of malignant tumour cells was only observed in two cases, indicating no crucial role in the occlusion of plastic stents (Weickert *et al.*, 2001). Similarly, a network of dietary fibres in biliary stents was identified by confocal laser scanning and scanning electron microscopy, which was assumed to act as sort of filter, contributing significantly to stent clogging (van Berkel *et al.*, 2005).

Even part of the natural defence system, the immunoglobulins (Ig) seem to play a role in the clogging process of biliary stents. Mainly IgA and IgG and minor amounts of IgM were observed in twelve blocked biliary stents deposited on the inner surface of the stents and mixed with clumps of bacteria (Chan *et al.*, 1998). Thus, rather than preventing bacterial colonisation of the biliary tree, the complex formation of IgA and IgG may accelerate clumping of bacteria and biofilm formation, thus contributing to the clogging process of biliary stents.

1.6 Goal of the study

In order to improve biliary stent lifetime and eliminate problems occurring during stent management it is important to gain a general understanding about the processes and events that lead to biofilm and sludge formation resulting in clogging of biliary stents. A fundamental knowledge on the formation, composition and persistence of the microbial community is therefore essential. However, so far, no detailed analysis of the microbial

community composition and their variability between different stents, patients or hospitals is available. To reach such fundamental knowledge, culture-independent methods were applied to describe the microbial community structure in biliary stents. In addition to information about the mere presence of microorganisms, the elucidation of possible metabolic activities in biliary stent biofilms was attempted, since they may contribute further to an accelerated occlusion of the stents.

2 Materials and methods

2.1 Bacterial strains and plasmids

Strains and plasmids used in the present study are summarised in table 2.1 and 2.2.

Table 2.1: Bacterial strains used in the present study.

Strain	Reference
Clostridium scindens DSM 5676	(Morris *et al.*, 1985)
Escherichia coli DH10B	Invitrogen
Escherichia coli JM 109	Promega
Lactobacillus plantarum 80 pCBH1	(Christiaens *et al.*, 1992)

Table 2.2: Plasmids used in the present study.

Plasmid	Reference
pGEM®-T	Promega
pGEM®-T Easy	Promega

2.2 Media and growth conditions

All listed media were sterilised by autoclaving for 20 min at 121 ℃ and 1 bar. Solid media were supplemented with 1.5 % (w/v) agar before autoclaving. Temperature sensitive components were filtered with a syringe filter of 0.2 µm pore size and added after autoclaving.

Brain heart infusion medium:

Brain heart infusion from solids	8	g
Peptic digest of animal tissue	5	g
Pancreatic digest of casein	16	g
Dextrose	2	g
NaCl	5	g
Na_2HPO_4	2.5	g
H_2O_{dest} ad	1000	ml

Columbia agar plates with 5 % sheep blood were purchased from BBL and contained:

Pancreatic digest of casein	12	g
Peptic digest of animal tissue	5	g
Yeast extract	3	g
Beef extract	3	g
Corn starch	1	g
NaCl	5	g
Sheep blood	5	%
H_2O_{dest} ad	1000	ml

Cooked meat medium:

Heart tissue granules	98	g
Peptic digest of animal tissue	20	g
Dextrose	2	g
NaCl	5	g
H_2O_{dest} ad	1000	ml

Luria-Bertani (LB) medium (Sambrook *et al*., 1989):

Yeast extrakt	5	g
Trypton	10	g
NaCl	10	g
H_2O_{dest} ad	1000	ml

SOC medium:

Trypton	2	g
Yeast extract	0.5	g
1M NaCl	1	ml
1M KCl	0.25	ml
2M Mg^{2+} stock solution	1	ml
2M Glucose	1	ml
H_2O_{dest} ad	100	ml

Trypticase soy medium:

Pancreatic digest of casein	17	g
Papaic digest of soybean meal	3	g
NaCl	5	g
K_2HPO_4	2.5	g
Dextrose	2.5	g
H_2O_{dest} ad	1000	ml

Anaerobic mineral medium supplemented with 20 g/l ox bile:

The mineral medium used for anaerobic cultivation contained 1 % (v/v) of solutions I and II and was supplemented with 0.2 % (v/v) of a vitamin and trace element (acidic and alkaline) stock solutions. This mixture was boiled for 10 minutes and then cooled under an inert gas mixture (N_2/CO_2–4:1). Then, $NaHCO_3$ as buffer (2.5 g/l), yeast extract (0.2–0.5 g/l), Na_2S x 9 H_2O as reducing agent (0.5 g/l) and bovine ox bile (Sigma-Aldrich) as supplement (20 g/l) were added. Resazurin was used as an indicator of anaerobic conditions (0.2 % (v/v) of stock solution). After distribution under inert gas to anaerobic cultivation bottles the medium was autoclaved.

Solution I:

NH_4Cl	33	g
$MgCl_2$ x $6H_2O$	50	g
$CaCl_2$	16.8	g
KCl	33	g
H_2O_{dest} ad	1000	ml

Solution II:

KH_2PO_4	33	g
H_2O_{dest} ad	1000	ml

Vitamin solution:

Biotin	10	mg
Folic acid	10	mg
Pyridoxine hydrochloride	50	mg
Riboflavine	25	mg
Thiamine	25	mg
Nicotinic acid	25	mg
Pantothenic acid	25	mg
Cyanocobalamin	10	mg
4-Aminobenzoic acid	25	mg
Lipoic acid	25	mg
H_2O_{dest} ad	1000	ml

Acidic microelement solution:

$FeSO_4$ x 7 H_2O	0.3	g
$CoCl_2$ x 6 H_2O	0.18	g
$MnCl_2$ x 4 H_2O	0.1	g
$ZnSO_4$ x 7 H_2O	0.15	g
$KAl(SO_4)_2$ x 12 H_2O	0.025	g
$NiCl_2$ x 6 H_2O	0.18	g
H_3BO_3	0.01	g
$CuCl_2$ x 2 H_2O	0.025	g
Adjust pH to 2 with HCl		
H_2O_{dest} ad	1000	ml

Alkaline microelement solution:

Na_2SeO_4	0.14	g
Na_2MoO_4 x 2 H_2O	0.06	g
Na_2WO_4 x 2 H_2O	0.17	g
Adjust pH to 10 with KOH		
H_2O_{dest} ad	1000	ml

Resazurin stock solution:

Resazurin	0.2	g
0,1N NaOH	20	ml
H_2O_{dest} ad	500	ml

Bile aesculin medium:

Peptone	8	g
Bile salts	20	g
Ferric citrate	0.5	g
Aesculin	1	g
H_2O_{dest} ad	100	ml

MacConkey medium:

Peptone	17	g
Proteose Peptone	3	g
Bile Salts	1.5	g
NaCl	5	g
Neutral Red	0.03	g
Crystal Violet	0.001	g
H_2O_{dest} ad	100	ml

De Man, Rogosa and Sharpe (MRS) medium:

Peptone	10	g
Lab-Lemco Powder	8	g
Yeast extract	4	g
Glucose	20	g
Tween 80	1	ml
K_2HPO_4	2	g
Sodium acetate x 3 H_2O	5	g
Triammoniumcitrate	2	g
$MgSO_4$ x 7 H_2O	0.2	g
$MnSO_4$ x 4 H_2O	0.05	g
H_2O_{dest} ad	100	ml

Additives:

Additive	Concentration stock solution	Solvent	Working concentration
Ampicillin	50 mg/ml	H_20	100 µg/ml
IPTG	25 mg/ml	H_2O	50 µg/ml
Taurodeoxycholic acid	50 mg/ml	H_2O	5 g/l
X-Gal	50 mg/ml	DMF	80 µg/ml

2.3 Chemicals

If not stated differently, all chemicals used in this study were supplied by AppliChem, BBL, Difco, Merck, ROTH or Sigma-Aldrich and were of the highest grade available.

2.4 Solutions

Listed are the compositions of all buffers and solutions, which are not further described in the text. Double distilled water was used for all preparations and dilutions.

SSCP loading dye

Formamide	9.5	ml
2M NaOH	50	µl
Bromphenolblue	2.5	mg
Xylenecyanol	2.5	m
ddH_2O ad	10	ml

SSCP elution buffer

10 mM Trisbase	0.157	g
5 mM KCl	0.037	g
1,5 mM $MgCl_2$ x 6 H_2O	0.030	g
0,1% TritonX 100	100	µl
Adjust pH to 9 with KOH		
ddH_2O ad	100	ml

6x DNA loading buffer

Xylenecyanol	0.125	g
Bromphenolblue	0.125	g
Glycerol	17	ml
ddH_2O ad	50	ml

50x TAE buffer

Tris/Acetate	242	g
Glacial acetic acid	57.1	ml
0,5M EDTA pH 8,0	100	ml
ddH_2O ad	1000	ml

10x TBE buffer

Tris	108	g
Boric acid	55	g
Na_2EDTA x 2H_2O	8.3	g
ddH_2O ad	1000	ml

1x TE buffer

1M Tris-HCl pH 8,0	5	ml
0,5M EDTA pH 8,0	1	ml
ddH_2O ad	500	ml

2.5 Biliary stent samples

Biliary stent samples were obtained from three different hospitals, the Surgery Clinic of Braunschweig (BS), the University Medical Centre of Schleswig-Holstein in Kiel (SH) and the Medical Clinic of Salzgitter-Lebenstedt (SZ). Samples intended for analysis

with molecular methods were placed at –20 °C directly after withdrawal and stored until processing. Prior to extraction of DNA, the outer surface of samples was wiped with 70% ethanol to minimise contamination resulting from the stent removal procedure. Biliary stent samples used for isolation of microorganisms were either transferred directly after collection to an anaerobic jar used with the AnaeroGen system (Oxoid, Cambridge, UK) to generate an anaerobic atmosphere or otherwise used only for isolation of aerobic microorganisms.

2.6 Isolation of microorganisms

2.6.1 Aerobic isolation procedures

For aerobic isolation of microorganisms 0.5 cm sections of both stent ends were vortexed separately in 1 ml of PBS and plated in serial dilutions on the following media: Mac Conkey, Bile Aesculin and Columbia Sheep Blood agar. Plates were incubated at 37°C and growing colonies purified by repeated subculturing and streaking on plates. For identification of isolated microorganisms 16S rRNA gene sequencing was used. DNA was isolated by boiling single colonies in TE buffer for 10 min, centrifugation and using the supernatant for 16S rDNA PCR. For gram-positive isolates DNA was extracted by the FastDNA Spin Kit for Soil (MP Biomedicals) according to the protocol of the manufacturer.

2.6.2 Anaerobic isolation procedures

For anaerobic isolation 0.5 cm sections of both stent ends were incubated separately in 20 ml of anaerobic mineral medium supplemented with 20 g/l ox bile. After 2 weeks incubation at 37 °C, 100 µl of this enrichment culture were plated and incubated under anaerobic conditions using the anaerobic working station MACS (Modular Atmosphere Controlled System) MG 500 (Meintrup DWS, Lähden). The following selective media were used for isolation: Mac Conkey, Bile Aesculin, Cooked Meat and MRS agar. Growing colonies were purified on selective media and further processed as described above for aerobic isolation procedures.

2.6.3 Strain maintenance

Stocks of isolated strains were prepared by supplementing 750 µl of cultures grown overnight in the appropriate liquid medium with 500 µl glycerol. Stocks were stored at –70℃ and strains streaked out on agar plates to che ck for purity before inoculating fresh liquid cultures.

2.7 Molecular techniques

Standard DNA manipulations were carried out essentially according to Sambrook *et al.* (Sambrook *et al.*, 1989).

2.7.1 Extraction of genomic DNA

Biliary stents were cut into sections and opened longitudinal. Approximately 3 cm of each stent side were used for extraction of DNA from the proximal and distal liver end, respectively. Samples were transferred into the lysing matrix tubes of the FastDNA Spin Kit for Soil (MP Biomedicals) and DNA extracted according to the protocol of the manufacturer.

2.7.2 Extraction of RNA

RNA from biliary stent samples was extracted using the FastRNA® Pro Blue Kit according to the manufacturers instructions. Briefly, the kit is based on a mechanical cell lysis in a solution that prevents RNA degradation. After release of cell contents the RNA is purified and isolated by chloroform extraction and ethanol precipitation.

2.7.3 Determination of nucleic acid concentration

2.7.3.1 Photometric determination

To determine the concentration of nucleic acids, the absorbance of diluted solutions was measured at 260 nm against the appropriate blank solution using an Eppendorf BioPhotometer. At that wavelength, an absorption of 1 corresponds to a concentration of 50 µg/ml DNA or 40 µg/ml RNA. The ratio of absorbance at 260 nm to absorbance at 280 nm gives an indication on the purity of DNA. For pure DNA, this ratio should be in the range of 1.8 - 2.

2.7.3.2 Determination using fluorescent dyes

Concentrations of double-stranded DNA, single-stranded DNA and RNA were determined using the fluorescent dyes PicoGreen®, OliGreen® and RiboGreen®, respectively (Invitrogen; Carlsbad, CA, USA) according to the protocol of the manufacturer. Reactions were carried out in black 96 well plates (Corning International; Corning, NY, USA) and measured with a Wallac Victor 2 Multi-label counter (Perkin-Elmer; Wellesley, MA, USA) at 480 nm excitation and 520 nm emission wavelengths. A standard curve prepared of λ DNA/Hind III digest (Invitrogen; Carlsbad, CA, USA), a 18-bp M13 sequencing primer (Invitrogen; Carlsbad, CA, USA) or 16S and 23S rRNA from *E. coli*, for double-stranded DNA, single-stranded DNA and RNA, respectively, was measured simultaneously with each assay to allow quantification.

2.7.4 Agarose gel electrophoresis

Horizontal agarose electrophoreses were applied for the analytical and preparative separation of DNA fragments in Gibco BRL chambers. Agarose concentrations were in the range of 0.5-2 % in 1 x TAE buffer according to the expected size and type of DNA fragments. 6 x loading buffer was added to the DNA samples before loading. Gels were run in 1 x TAE buffer applying voltages between 20–110 Volt. Staining was performed in an 1 µg/ml ethidium bromide solution in water for 10 min. Pictures were taken with a CCD camera (Herolab, Modell 429 K) on a transilluminator at 312 nm (Herolab, Modell

UVP). For documentation pictures were printed with a video printer (Mitsubishi Video Copy Processor).

2.7.5 Polymerase chain reaction (PCR)

The polymerase chain reaction is an automated *in vitro* method, which allows a targeted amplification of DNA fragments from DNA templates using oligonucleotides as primers. Taq DNA polymerase of Qiagen (Hilden, Germany) was used for all PCR reactions. Synthetic oligonucleotides were provided by Invitrogen or Operon of purity grade desalted and are listed in table 2.3.

Table 2.3: Synthetic oligonucleotides used in this study.

Name	Sequence 5'-3'	Length (bp)	Annealing Temp. ℃	Application	Reference
Com I	CAGCAGCCGCGGTAATAC	18	50, 58, 60	PCR, sequencing	(Schwieger & Tebbe, 1998)
Com II	CCGTCAATTCCTTTGAGTTT	20	50, 58, 60	PCR (Reamplification of SSCP bands)	(Schwieger & Tebbe, 1998)
Com II - P	P-CCGTCAATTCCTTTGAGTTT	20	50, 58, 60	PCR, sequencing	(Schwieger & Tebbe, 1998)
16S F27	AGAGTTTGATCMTGGCTCAG	20	60	PCR	(Lane, 1991)
16S R1492	TACGGYTACCTTGTTACGACTT	22	60	PCR	(Lane, 1991)
16S F357	ACTCCTACGGGAGGCAGCAG	20	60	Sequencing	Modified after (Lane, 1991)
16S F945	GGGCCCGCACAAGCGGTGG	19	60	Sequencing	Modified after (Lane, 1991)
16S R518	CGTATTACCGCGGCTGCTGG	20	60	Sequencing	Modified after (Lane, 1991)
16S R1087	CTCGTTGCGGGACTTAACCC	20	60	Sequencing	Modified after (Lane, 1991)

M13 f	GTTTTCCCAGTCACGAC	17	60	PCR, sequencing	Promega
M13 r	CAGGAAACAGCTATGAC	17	60	PCR, sequencing	Promega
Bai CD-F	GGWTTCAGCCCRCAGATGTTCTTTG	25	52	PCR	(Wells *et al.*, 2003)
Bai CD-R	GAATTCCGGGTTCATGAACATTCTKCKAAG	30	52	PCR	(Wells *et al.*, 2003)
TPA-F	CAACGTCCCCACCATCAAGTTCTCTG	26	65	PCR	(Laue *et al.*, 2006)
TPA-R	TGAATTCGCGGAAGGAGCGAGAGGTC	26	65	PCR	(Laue *et al.*, 2006)

2.7.5.1 Com PCR

The Com PCR was described by Schwieger and Tebbe for amplification of an approximately 370 bp fragment of the 16S rDNA enclosing the variable regions IV and V using universal eubacterial primers (Schwieger & Tebbe, 1998). The Com primers (see table 2.3) correspond to the *E. coli* positions 519-536 and 907-926 of the 16S rRNA gene. The reverse primer Com II was used phosphorylated at the 5' end, when PCR products were intended for Single-Strand-Conformation Polymorphism (SSCP) analysis. The composition of the PCR reaction mix and the thermocycler program are listed in tables 2.4 and 2.5.

Table 2.4: Com PCR reaction mix.

Component	Conc. stock	Volume added	Final conc.
Primer Com I	10 mM	1.5 µl	300 µM
Primer Com II – P	10 mM	1.5 µl	300 µM
dNTPs	1.25 mM	2 µl	50 µM
$MgCl_2$	10 mM	2 µl	400 µM
PCR buffer	10 x	5 µl	1 x
Qiagen Taq polymerase	5 U/µl	0.5 µl	2.5 U
DNA template	10 ng/µl	1 µl	10 ng
Milli Q H_20 ad. 50 µl			

Table 2.5: PCR thermocycler program.

Step	Temperature	Time
Initial denaturation	95 ℃	3 min
Denaturation	94 ℃	40 sec
Annealing	50 or 58 ℃	40 sec
Elongation	72 ℃	1 min
Final elongation	72 ℃	10 min
Cooling and hold	4℃	∞
Number of cycles	30 x	

2.7.5.2 Reverse Transcriptase Com PCR

A Reverse Transcriptase (RT) Com PCR was performed directly with 1µl of extracted RNA using the *C. therm.* polymerase one-step RT-PCR system of Roche (Penzberg, Germany), which is composed of an enzyme mix containing the Klenow fragment of DNA polymerase from *Carboxydothermus hydrogenoformans* and the thermostable Taq DNA polymerase. The composition of the PCR reaction mix and the thermocycler program are listed in tables 2.6 and 2.7.

Table 2.6: Reverse Transcriptase Com PCR reaction mix.

Component	Conc. stock	Volume added	Final conc.
Primer Com I	10 mM	2 µl	400 µM
Primer Com II – P or Com II	10 mM	2 µl	400 µM
dNTPs	25 mM	0.8 µl	400 µM
DMSO	100 %	2.5 µl	7 %
DTT	100 mM	2.5 µl	5 mM
RNase inhibitor (Ambion)		1 µl	
RT-PCR buffer	5 x	5 µl	1 x
C. therm. polymerase mixture		2 µl	
RNA template		1 µl	
Milli Q H_20 ad. 50 µl			

Table 2.7: Reverse Transcriptase Com PCR thermocycler program.

Step	Temperature	Time
RT step	60 ℃	30 min
Initial denaturation	94 ℃	3 min
Denaturation	94 ℃	40 sec
Annealing	50 ℃	40 sec
Elongation	72 ℃	1 min
Final elongation	72 ℃	10 min
Cooling and hold	4℃	∞
Number of cycles	35 x	

2.7.5.3 16S rDNA PCR

For generation of 16S rDNA clone libraries or the identification of bacterial isolates based on 16S rRNA gene sequences, a PCR using the universal eubacterial primers 16S F27 and 16S R1492 (see table 2.3) was performed. The composition of the PCR reaction mix and the thermocycler program are listed in tables 2.8 and 2.9.

Table 2.8: 16S rDNA PCR reaction mix.

Component	Conc. stock	Volume added	Final conc.
Primer 16S F27	10 mM	1 µl	200 µM
Primer 16S R1492	10 mM	1 µl	200 µM
dNTPs	1.25 mM	4 µl	100 µM
PCR buffer	10 x	5 µl	1 x
Qiagen Taq polymerase	5 U/µl	0.5 µl	2.5 U
DNA template	10 ng/µl	1 µl	10 ng
Milli Q H_20 ad. 50 µl			

Table 2.9: 16S rDNA thermocycler program.

Step	Temperature	Time
Initial denaturation	94 ℃	1 min
Denaturation	94 ℃	10 sec
Annealing	60 ℃	20 sec
Elongation	72 ℃	1 min
Final elongation	72 ℃	3 min
Cooling and hold	4℃	∞
Number of cycles	30 x	

2.7.5.4 Taurine–pyruvate aminotransferase PCR

In order to check for the presence of the bacterium *Bilophila wadsworthia*, a specific PCR targeting the taurine–pyruvate aminotransferase of this bacterium was conducted according to the protocol of Laue *et al.* (Laue *et al.*, 2006). The composition of the PCR reaction mix and the thermocycler program are listed in table 2.10 and 2.11. Primer sequences are listed in table 2.3.

Table 2.10: Taurine–pyruvate aminotransferase PCR reaction mix.

Component	Conc. stock	Volume added	Final conc.
Primer TPA-F	10 mM	1.5 µl	300 µM
Primer TPA-R	10 mM	1.5 µl	300 µM
dNTPs	1.25 mM	4 µl	100 µM
PCR buffer	10 x	5 µl	1 x
Qiagen Taq polymerase	5 U/µl	0.5 µl	2.5 U
DNA template	10 ng/µl	1 µl	10 ng
Milli Q H_20 ad. 50 µl			

Table 2.11: Taurine-pyruvate aminotransferase thermocycler program.

Step	Temperature	Time
Initial denaturation	94 ℃	5 min
Denaturation	94 ℃	30 sec
Annealing	65 ℃	30 sec
Elongation	72 ℃	2 min
Final elongation	72 ℃	7 min
Cooling and hold	4℃	∞
Number of cycles	30 x	

2.7.5.5 Bile acid inducible (bai) operon PCR

To demonstrate the presence of the bile acid inducible (bai) operon, coding for genes involved in the 7α-dehydroxylation of free bile acids, a PCR was conducted according to the protocol of Wells et al. (Wells *et al.*, 2003). Extracted DNA of biliary stent communities in different dilutions was subjected directly to PCR. DNA of *Clostridium scindens* DSM5676 was used as positive control in concentrations of 2 and 20 ng. Furthermore, stent community DNA was spiked with 2 and 20 ng of *Clostridium scindens* DSM5676 DNA to check for the inhibitory influence of the DNA extracts on PCR reactions.

Table 2.12: Bai operon PCR reaction mix.

Component	Conc. stock	Volume added	Final conc.
Primer bai CD-F	10 mM	2.5 µl	500 µM
Primer bai CD-R	10 mM	2.5 µl	500 µM
dNTPs	1.25 mM	8 µl	200 µM
$MgSO_4$	10 mM	7.5 µl	1.5 mM
DMSO		2.5 µl	5 %
BSA	10 mg/ml	1 µl	10 µg
PCR buffer	10 x	5 µl	1 x
Qiagen Taq polymerase	5 U/µl	0.5 µl	2.5 U
DNA template	10 ng/µl	1 µl	10 ng
Milli Q H_20 ad. 50 µl			

Table 2.13: Bai operon PCR thermocycler program.

Step	Temperature	Time
Initial denaturation	94 °C	2 min
Denaturation	94 °C	20 sec
Annealing	52 °C	30 sec
Elongation	69 °C	1.5 min
Final elongation	68 °C	10 min
Cooling step	4°C	∞
Number of cycles	35 x	

2.7.6 M13 PCR

The M13 PCR was used to amplify PCR fragments cloned in the pGEM®-T or pGEM®-T Easy vector for sequencing. The composition of PCR reactions and the thermocycler program were as described above for 16S rDNA PCRs. Template DNA was extracted directly from recombinant colonies by transferring them in 100 µl TE buffer, boiling at 96 °C for 10 min followed by centrifugation. 1 µl of the supernatant was used as template DNA.

2.7.7 Purification of nucleic acids

2.7.7.1 Purification of PCR products

PCR products were either purified with the Qiagen MinElute or the Qiagen QIAquick kit, depending on the designated elution volume (10-20 µl with the MinElute and 30-50 µl with the QIAquick kit). In case PCR products had been prepared in 96-well plates, they were purified using the Machery-Nagel NucleoFast 96 PCR purification kit. All kits were used according to the instructions of the manufacturers.

2.7.7.2 Extraction of DNA from agarose gels

DNA from agarose gels was purified using the Geneclean SPIN Kit of MP Biomedicals according to the instructions of the manufacturer.

2.7.8 Single-Strand-Conformation Polymorphism (SSCP) fingerprinting

Single-Strand-Conformation Polymorphism (SSCP) is a technique that was originally developed for point mutation analysis in medicine (Orita *et al.*, 1989). In a modified form it can be also used as a fingerprinting method in microbial community analysis, comparable to DGGE or TGGE. The separation principle is based on different migration properties of single-stranded DNA, dependent on spatial structures. The method was used as described by Schwieger (Schwieger & Tebbe, 1998).

2.7.8.1 Enzymatic digestion of double stranded DNA

Purified Com PCR products (see 2.7.7.1) were subjected to a lambda exonuclease digestion. The phosphorylated 5' end of generated PCR products is recognised by the enzyme converting the PCR product into single stranded DNA. The digest was conducted at 37 ℃ for 1 hour in a volume of 20 µl, using 2.5 µl of enzyme (12.5 U) and 2.5 µl of the corresponding buffer (New England Biolabs). Subsequently, reaction mixtures were purified using the MinElute Kit (Qiagen) and single stranded DNA was quantified with Oli Green (see section 1.7.3.2.).

2.7.8.2 SSCP gel and running conditions

An aliquot containing 50-100 ng of single-stranded DNA was dried in an Eppendorf SpeedVac Concentrator 5301 and resuspended in 6 µl of 1:2 diluted SSCP loading dye. Samples were heated to 96℃ for 3 min to denat ure DNA and immediately placed on ice for approximately 5 minutes to allow formation of single-stranded DNA conformations. Samples were then loaded onto a 0,6x MDE polyacrylamide gel. The SSCP system (2010-001 Macrophor Electrophoresis Unit, LKB Bromma) was kept at a constant temperature of 20 ℃, to ensure defined sp atial structures of the single-stranded DNAs. Running parameters were 400 V for 16 hours at a constant temperature of 20 ℃.

2.7.8.3 Silver staining and documentation

For detection of nucleic acids, gels were silver stained as reported previously (Bassam *et al.*, 1991). For documentation purposes, images were taken on C/RA duplication films (Kodak) and gels were scanned with a transmitted light scanner with a resolution of 300 dpi. Scanned gels were normalised according to the marker lane using the program GelCompar II (Applied Maths, Belgium). The marker corresponded to a mixture of single stranded DNA generated from bacterial isolates (*Klebsiella oxytoca*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Agrobacterium radiobacter*, the first 3 being biliary stent isolates) and was loaded onto the two margins and in the centre of the gel. Dr. Frank Schwieger is acknowledged for providing DNA of the *Agrobacterium radiobacter* isolate.

2.7.8.4 Excision of SSCP bands

Prior to excision of bands, the gel was wiped with 70% ethanol and placed onto a light table. Bands were excised with a sterile scalpel and transferred to 30 µl SSCP elution buffer. Samples were then boiled for 10-15 min in a water bath at 95 °C. After centrifugation for 1 min, 1 µl of the supernatant was used for reamplification in a Com PCR using an annealing temperature of 58 °C. Sequencing was performed as described in chapter 2.7.10.

2.7.8.5 Analysis of SSCP fingerprint data

A SSCP phylotype is defined as one or several bands only showing up concurrently and having a highly similar sequence (≥ 99% sequence similarity). As comparisons between gels were limited due to slight differences in migration behaviour of single-stranded DNA on different gels, a SSCP band was only assigned to the respective phylotype when it was either sequenced or showed a migration behaviour identical to that of another band analysed on the same gel from which sequence information had been obtained. Absence and presence of SSCP phylotypes from each fingerprint was converted to an absence/presence table, later used for multivariate statistical analysis. The prevalence of SSCP phylotypes from BS and SZ hospitals were analysed separately. To compare the community composition of stents originating from each

hospital, the combined abundances of SSCP phylotypes across all samples were compared at the family and the phylum level.

Non-parametric multivariate statistical analysis was performed using PRIMER v6 (Plymouth Marine Laboratory, UK) (Clarke, 1993). The multivariate routines applied here were non-metric multidimensional scaling (nMDS), analysis of similarity (ANOSIM), similarity percentage analysis (SIMPER) and dispersion analysis, as described in detail by Wilber et al. (Wilber *et al.*, 2007). Sample similarity matrices were calculated from absence/presence data using a Bray-Curtis coefficient. Non-metric multidimensional scaling (nMDS) was used to ordinate each sample based on its microbial community structure, thus visualising the similarity and dissimilarity between each sample. The program used 50 random restarts to find the optimal ordination and stress value. The stress value of each nMDS plot represents an estimation of the quality of fit of the data. A stress value below 0.1 corresponds to an ideal ordination indicating that there is no real prospect of misinterpretation, a stress value below 0.2 corresponds to a useful ordination indicating a valuable 2D representation and a stress value higher than 0.2 indicates that the plot is close to random (Clarke & Warwick, 2001). With more samples being placed on a plot the stress value tends to increase. However, if there are many samples on a plot and the stress value is still reasonable (<0.2), the information cannot be misinterpreted.

Analysis of similarity (ANOSIM) was used to test for significant differences between predefined groups of samples (e.g. from the same patient). It produces a test statistic (R) which can range from –1 to 1 and is a useful comparative measure of the degree of separation between groups. A R-value higher than 0 indicates that samples are more different between groups than within groups. A R-value of 0 indicates the null hypothesis is true, and that there are no significant differences between groups. R usually falls between 0 and 1 which indicates some degree of distinction between groups. A R-value lower than 0.25 indicates barely separable groups, a R-value higher than 0.5 indicates clear differences between groups with some degree of overlap, and a R-value higher than 0.75 indicates well separable groups (Wilber *et al.*, 2007). When testing more than two groups a global R test indicates if differences between groups are present that may be worth examining further. If the Global R is not significant, generally no further interpretation is permissible. If it is significant, R-values for each pairwise comparison should be examined.

Similarity percentages analysis (SIMPER) was used to determine which of the SSCP phylotypes mostly contribute to the similarity and dissimilarity between groups of samples originating from the same patients. This identifies the most discriminating SSCP phylotypes (Clarke, 1993). SIMPER was performed until more than 90% of all similarity or dissimilarity within or between groups was accounted for, and of the identified SSCP phylotypes those contributing more than 10% were included in the analysis.

Dispersion analysis was used to calculate the closeness of grouping among samples originating from the same patient. Dispersion analysis compares the average rank similarity across a given defined group of samples. A low dispersion index indicates a tight grouping of samples, while a higher value indicates larger within group heterogeneity (variability).

2.7.9 Cloning of PCR products

Cloning of PCR products was performed with the pGEM®-T or pGEM®-T Easy vector system (Promega, Mannheim). The vectors are linearised and have a 3' terminal thymidine at both ends to prevent recircularisation. The compatible overhang of PCR products is generated by the Taq polymerase, which adds a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments. The high copy number vectors contain M13 forward and reverse primer binding sites, which flank the multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase. The selection marker used is an ampicillin resistance cassette.

2.7.9.1 Ligation

Purified PCR products were ligated into the vector using the T4 ligase of Fermentas (3 Weiss units). A molar ratio of 3:1 of insert DNA to vector DNA was used for all ligations. Ligations were performed in 10 µl reactions at 4℃ over night.

2.7.9.2 Transformation

Transformations were performed with the heat shock method using 2 µl of the ligation and 50 µl of chemically competent *Escherichia coli* DH10B or *Escherichia coli* JM 109 cells (see table 1.1). Cells were incubated on ice for 20 min, followed by a heat shock for 45-50 sec at 42 ℃. After 2 min incubation on i ce, cells were incubated for 1.5 hours at 37℃ and 150 rpm in 900 µl SOC medium. 100 µl of each transformation were then plated in duplicates on LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37℃.

2.7.9.3 Blue-white selection

To select for antibiotic resistant clones that carry a vector with insert, the blue-white screening system was used. The multiple cloning site of the vector system is located inside the *lacZ* gene. In case no insert is present, the inactive β-galactosidase of host strains is complemented by the α-peptide of the vector. This complementation is detected by cleavage of the substrate X-Gal in presence of the inducer IPTG, resulting in the blue dye 5-bromo-4-chloro indigo. Accordingly, only white colonies were picked from the selective plates and used for further analysis.

2.7.10 Sequencing of DNA

Sequencing was performed on an ABI PRISM™ 3100 Genetic Analyser (Applied Biosystems, Darmstadt). The reaction is based on the chain-terminating inhibitors method of Sanger *et al.* (Sanger *et al.*, 1977) and fluorescent labelled terminators (ddNTP) were used.

2.7.10.1 Sequencing reaction

Sequencing reactions were performed with the BigDye Terminator™ v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt). The BigDye ready reaction mix contains the terminators, dNTPs, $MgCl_2$ and *AmpliTaq* ® DNA polymerase FS.

Table 2.14: Sequencing reaction mix.

Component	Conc. stock	Volume added	Final conc.
Sequencing primer	10 mM	0.5 µl	100 µM
BigDye buffer	5.0 x	1 µl	0.5 x
BigDye ready reaction mix	2.5 x	2 µl	0.5 x
DNA template		1 – 6.5 µl	
Milli Q H_20 ad. 10 µl			

Table 2.15: Sequenching reaction thermocycler program.

Step	Temperature	Time
Initial denaturation	96 ℃	1 min
Denaturation	96 ℃	10 sec
Annealing	60 ℃	5 sec
Elongation	60 ℃	4 min
Cooling and hold	4℃	∞
Number of cycles	25 x	

2.7.10.2 Purification and sequencing work

After the sequencing reaction, excess dNTPs were removed with the Dye Ex™ Spin Kit (Qiagen, Hilden) according to the protocol of the manufacturer. Samples were dried in an Eppendorf Speed Vac Concentrator and stored at –20℃ until further usage. For sequencing, samples were resuspended in 20 µl highly deionised Hi-Di™ formamide (Applied Biosystems, Darmstadt) before electrophoresis. All sequencing work with the ABI PRISM™ 3130 xl Genetic Analyser was kindly performed by Annette Krüger or Julia Strömpl in the microbiology department of the Helmholtz Centre for Infection Research.

2.8 Sequence data analysis

Sequencing trace files were checked for quality and aligned using the software Sequencher (version 4.0.5; Gene Codes Corporation) or SeqAssem (version 01/2005; Dominik Hepperle). After removal of primer sequences, sequences were classified by

comparison to public databases using the BLAST algorithm at NCBI (Altschul *et al.*, 1990). 16S rDNA sequences of bacterial isolates were uploaded to the Ribosomal Database Project II (release 9.49) (Cole *et al.*, 2007), aligned to the database and classified using the Sequence Match tool (version 3, Release 9.49, April 2007).

Almost full length 16S rDNA sequences originating from random sequencing of 16S rDNA clone libraries were checked for chimera or other sequence anomalies using Mallard (Ashelford *et al.*, 2006) and Bellerophon (Huber *et al.*, 2004). Following alignment of the remaining sequences using the CLUSTAL X program, a distance matrix was calculated with the DNADIST program of the PHYLIP package (version 3.66) according to the Jukes-Cantor model. Operational Taxonomic Units (OTUs) at cut-off levels of 1.5% and 2% sequence difference were then defined using the program DOTUR (Schloss & Handelsman, 2005) with the furthest neighbour algorithm. Chao-1 richness estimators (Chao, 1987) and rarefaction analyses were used to estimate species richness within microbial communities of biliary stent biofilms. In addition, to estimate the diversity of communities, diversity indices were calculated using DOTUR (Schloss & Handelsman, 2005). The Shannon index (H') (Magurran, 1988) is a general diversity index that takes into account the number of OTUs and the evenness, and is defined as:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of OTUs and p_i is the relative abundance of each OTU, calculated as the proportion of clones of a given OTU (n_i) to the total number of sequenced clones (N): $\frac{n_i}{N}$ (Magurran, 1988).

The Simpson index (D) (Magurran, 1988) is a dominance measure which represents the probability that two randomly selected sequences belong to the same OTU, and is defined as:

$$D = \frac{\sum_{i=1}^{S} ni(ni-1)}{N(N-1)}$$

where S is the number of OTUs, n is the number of clones of a given OTU and N is the total number of sequenced clones (Magurran, 1988). The use of the reciprocal ensures that the value of the index rises with increasing diversity (Magurran, 2005).

Phylogenetic trees were generated using the software MEGA3 (Kumar *et al.*, 2004) by neighbour-joining analysis using a Jukes-Cantor correction. For nearly full length 16S rDNA sequences of the clone libraries pairwise deletion of gaps and for SSCP phylotypes and their reference sequences complete deletion of gaps was applied.

2.9 Biochemical methods

2.9.1 Screening for bile salt hydrolase (bsh) activity

Biliary stent isolates were tested for bile salt hydrolase activity with a plate assay (Dashkevicz & Feighner, 1989), which uses the conversion of taurodeoxycholic acid to deoxycholic acid and its following precipitation in an acidic environment. MRS agar plates were prepared without and with the supplement of 0.1 or 0.5% taurodeoxycholic acid and the pH was adjusted to 6.5. Plates were incubated at 37℃ in an anerobic chamber for three days and checked every 24 hours for growth and precipitation of deoxycholic acid. The strain *Lactobacillus plantarum* 80 (pCBH1) was used as positive control. This strain was kindly supplied by professor Willy Verstraete.

2.10 Transmission electron microscopy

Biliary stent biofilm matrices of the proximal and distal liver end were portioned and fixed in 2.5 % glutaraldehyde in 20 mM HEPES buffer, pH 7 at 4℃ and room temperature for 2 hours up to several days. After washing twice in 1 ml 100 mM sodium acetate buffer, pH 4 at room temperature for 15 min, the biofilm matrix was contrasted for acidic groups for 14 hours at 4℃ with 0.4 % (w /v) ThO_2 in 100 mM sodium acetate buffer, pH 4. After washing twice with 100 mM HEPES, pH 4 for 15 min, the material was dried by washing with aqueous solutions containing increasing amounts of ethanol (each step for 30 min at ambient temperature with concentrations of 10 %, 30 %, 50 %, 70 %, 100 % ethanol, followed by 60 min in 100 % ethanol). At the 70 % ethanol step on-bloc staining was performed for 60 min at room temperature with 1 % (w/v) UO_2 acetate. Incubation for 1 h at room temperature in a Spurr-epoxy hard resin -ethanol - mix (1+1) (Spurr, 1969) was followed by further incubation for 1 h in a 2+1 epoxy resin - ethanol mix. After incubation overnight in pure epoxy resin, samples were incubated for further 3 h at room temperature in fresh pure epoxy resin and afterwards

polymerised for 8 h at 70℃ in a flat bed mold. Ult rathin sections of 90 nm thickness were cut with a diamond knife (Diatome, Swiss), mounted in a Reichert-Jung Ultramicrotome, (Leica, Vienna, Austria) and were poststained with 2 % (w/v) UO_2 acetate, pH 4.5 and 0.1 % (w/v) lead citrate, pH 8.5 (Reynolds, 1963) and analysed in an energy-filter transmission electron microscope (CEM902, Zeiss, Oberkochen) at 12000 fold to 50000 fold magnification in the elastic brightfield mode. Electron micrographs were registered with a 1024 x 1024 CCD camera (ProScan, CCD HSS 512/1024; Proscan Electronic Systems, Scheuring) as 8 bit grey scale images. Sample preparation and electron microscopy was kindly performed by Inge Kristen and Dr. Heinrich Lünsdorf in the microbiology department of the Helmholtz Centre for Infection Research.

3 Results

3.1 Origin of biliary stents

Biliary stents were obtained from three different hospitals, the Surgery Clinic of Braunschweig (herein abbreviated to BS), the University Medical Centre of Schleswig-Holstein in Kiel (herein abbreviated to SH) and the Medical Clinic of Salzgitter-Lebenstedt (herein abbreviated to SZ). All three hospitals are characterised by differences in stent management. Whereas at SH biliary stents are exchanged in a regular, prophylactic manner after 2-3 months in order to prevent predictable problems due to clogging of the stents, which is typically followed by jaundice and cholangitis, at SZ and BS stents are only exchanged when problems, such as clogging or dislocation, occur or in case histology samples have to be taken. One additional difference between the hospitals is that the stents are either implanted in parallel or in succession. At SZ, several stents (up to three) are inserted simultaneously into the biliary and/or pancreatic ducts in order to prolong the time until complete clogging of the stents. In contrast, at BS clogged stents are replaced by a new stent, resulting in several stents from each patient over time. Moreover, at SZ it is common practice to treat patients in an outpatient procedure, while at BS patients are generally hospitalised. In all hospitals antibiotics are given to some patients to minimise the risk of cholangitis. All hospitals use representatives of the same antibiotic classes, namely 2^{nd} generation fluorochinolones and beta-lactames, but only BS uses nitroimidazol which is active against anaerobic bacteria (Table 3.1).

Table 3.1: Antibiotic use in the three different hospitals, Surgery Clinic of Braunschweig (BS), University Medical Centre of Schleswig-Holstein in Kiel (SH) and Medical Clinic of Salzgitter-Lebenstedt (SZ).

Antibiotic	Class	Mode of action	Target	Hospital
Ofloxacin	2nd generation Fluorchinolon	Gyrase inhibitor	Aerobic gram positive and negative bacteria	SZ
Ciprofloxacin	2nd generation Fluorchinolon	Gyrase inhibitor	Broad spectrum of bacteria, particularly gram negative	BS, SH
Ceftriaxon	Beta-Lactam antibiotic, Cephalosporin	Inhibitor of cell wall synthesis	Proliferating gram positive (not Enterococci) and gram negative bacteria	SZ
Sultamicillin (Ampicillin plus Sulbactam linked as a double ester)	Beta-Lactam antibiotic, Penicillin; Beta-Lactamase – Inhibitor	Inhibitor of cell wall synthesis	Gram positive and gram negative bacteria	BS, SH
Imipenem Cilastatin	Beta-Lactam antibiotic, Carbapenem; Inhibitor of Imipenem degradation	Inhibitor of cell wall synthesis	Gram positive and gram negative bacteria, aerobe and anaerobe; Not active against: *Enterococcus faecium*, *Pseudomonas* sp., *Clostridium difficile*	BS
Metronidazol	Nitroimidazol	Causes DNA strand breaks	Anaerobes, Protozoa	BS

3.2 Placement and removal of biliary stents by means of endoscopic retrograde cholangiopancreatography (ERCP)

All investigated stents were placed and removed by endoscopic retrograde cholangiopancreatography (ERCP). The endoscope consists of a flexible tube equipped with a small light source and a video camera. The endoscope is lowered down the oesophagus, through the stomach and into the duodenum. At the site of the major duodenal papilla (papilla of Vater), a cannula is threaded down through the endoscope and can be directed into the biliary or pancreatic duct. A liquid contrast solution is injected through the cannula backwards (retrograde) into the ducts giving

the possibility to take X-rays of the biliary and pancreatic system. This enables the gastroenterologist to identify narrowing or blockage of the ducts. If necessary, stents are placed through the endoscope. If a stent has to be removed, it is fetched with a wire sling and removed simultaneously with the endoscope, but remains outside the endoscopic channel. Thus, from the scientific viewpoint, the removal poses the risk of microbial contamination of the stents with microorganisms of the upper small intestine, the oesophagus and the oral cavity. On the other hand, during placement, contamination of the stent by microorganisms originating from the duodenum is possible.

3.3 Biliary stent microbial community composition analysed by SSCP fingerprinting

To obtain an overview on the composition and diversity of microbial community structures of biliary stents, a total of 64 stents, comprising 59 biliary and 5 pancreatic stents, from two different hospitals was analysed by SSCP fingerprinting. For biliary stents each a fingerprint for the stent end proximal and distal to the liver was generated to check for differences in community structures along the stent. For stents showing a biofilm visible by eye on the exterior of the proximal liver end, additional fingerprints of the exterior biofilm were generated to check for differences in community structures of inner and outer biofilms. Thus, in total 133 SSCP fingerprints were generated from the 64 stents analysed. A SSCP band or several bands, only showing up concurrently and having a highly similar sequence (≥ 99% sequence similarity) were grouped in SSCP phylotypes. SSCP fingerprint bands were only assigned to the respective phylotypes when they were either sequenced or showed identical migration behaviour to that of another sample analysed on the same gel that had been sequenced. Due to slight differences in migration behaviour of single stranded DNA on different gels, comparisons between gels were limited. Although comparability of different gels has been improved by the development of a single-stranded DNA marker, clear assignment of bands, which have not been sequenced from the same gel, to a phylogenetic group remains difficult and risky. In a few cases, repeated reamplification and sequencing of some bands did not result in unambiguous sequences. This is probably due to multiple sequences that are contained in a single band, as previously observed (Schmalenberger & Tebbe, 2003). Thus, these bands could not be assigned to any phylogenetic group. As evident from SSCP fingerprints presented in figures 3.2 and 3.3

more than 90% of the predominant species of all stents could successfully be assigned to a SSCP phylotype. In some other cases, closely migrating single-strand products represented identical ssDNA sequences, suggesting that the same single strand formed two conformers migrating with slightly different motilities. Accordingly, multiple bands on PCR-SSCP fingerprints of bacterial pure cultures were previously described (Schmalenberger *et al.*, 2001). Such double bands were thus regarded to represent one phylotype. Moreover, several bands with a highly similar, but not identical sequence can represent one microorganism, since rRNA operon copy numbers may vary from 1 to 15 among eubacterial genomes (Klappenbach *et al.*, 2001). The different copies may display micro-heterogeneity in their sequence, e.g. for *Veillonella* sp. four rRNA operon copies were described that show 98,5-100% of sequence similarity to each other (Marchandin *et al.*, 2003). Similarly, in this study for *Veillonella* sp. four bands, showing a similar but not identical sequence were observed on SSCP fingerprints, and thus summarised in SSCP phylotype 41. For the majority of SSCP phylotypes, the retrieved sequence information allows a classification down to the species level, however, in some cases classification was only possible down to the genus level.

3.3.1 SSCP phylotypes detected in biliary stents from Surgery Clinic of Braunschweig and Medical Clinic of Salzgitter

An overall of 62 bacterial SSCP phylotpyes could be detected in the total of 133 fingerprints obtained from stents of BS and SZ and an overview of the presence and abundance is given in figure 3.1. For each SSCP phylotype the representative sequence and either one or two most related reference sequences are included in the phylotree to allow orientation in the phylogeny of the domain bacteria. The presence of 6 bacterial phyla was observed. The most highly represented phylum was that of the Firmicutes and sequence analysis revealed 411 of the total 769 bands detected to originate from the presence of such microorganisms. An overall of 31 distinct phylotypes could be differentiated among the Firmicutes. The most abundant phylotype within the Firmicutes could be identified as *Veillonella* sp. (SSCP phylotype 41) which was detected in 83 of the 133 fingerprints, and is as well the most abundant phylotype of all. Other abundant phylotypes of the Firmicutes are SSCP phylotype 35 *Streptococcus anginosus* (detected in 64 fingerprints), SSCP phylotype 43 *Enterococcus faecalis* (detected in 48 fingerprints) and SSCP phylotype 44 *Enterococcus faecium* (detected in 45 fingerprints). The second most represented

phylum is that of the Proteobacteria, 15 distinct phylotypes were observed and 158 of the total 769 bands were due to the presence of members of this phylum. The most abundant proteobacterial phylotype was SSCP phylotype 23 *Escherichia coli* detected in 45 fingerprints. Organisms affiliated with the Fusobacteria phylum were observed in 80 of the total 769 bands and differentiated into 4 distinct phylotypes, the most abundant being SSCP phylotye 54 *Fusobacterium nucleatum* (B) (detected in 50 fingerprints). Another represented phylum is the Bacteroidetes phylum observed in 38 of the total 769 bands and comprising 8 distinct phylotypes. The Actinobacteria phylum was represented by 73 of the total 769 bands observed, which could be differentiated in 3 distinct phylotypes, the most abundant being SSCP phylotype 26 (*Bifidobacterium* sp.) detected in 67 fingerprints, this is as well the second most abundant of all phylotypes. The Deferribacteres phylum was only represented by the SSCP phylotype 10 (*Synergistes* sp.) and detected on 9 fingerprints. Furthermore, 16 of the total 769 bands showed a high homology to human DNA sequences (SSCP phylotype A) and 15 out of the total 769 bands observed were closely related to yeast sequences (*Candida albicans*, *Candida glabrata* or *Saccharomyces cariocanus*) and were designated SSCP phylotype B.

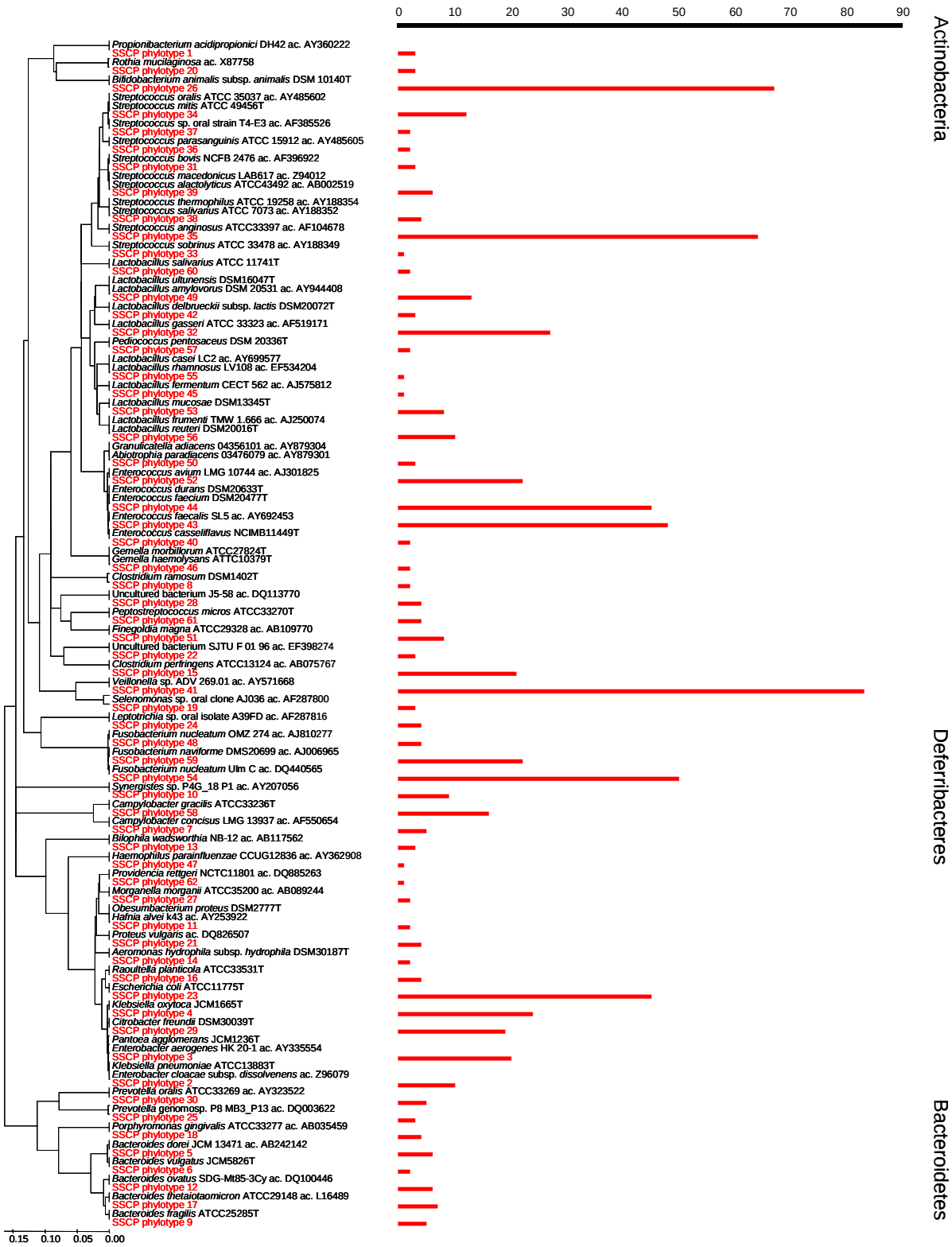

Figure 3.1: Phylogenetic tree showing the affiliation of bacterial SSCP phylotypes and related reference strains. The tree was constructed by neighbour-joining analysis using a Jukes-Cantor correction. The scale bar represents the genetic distance (0.1 corresponds to 10 substitutions per 100 nucleotides). The right side of the figure shows the abundance of the SSCP phylotypes and the phyla the sequences are affiliated with. Ac: accession number.

3.3.2 Surgery Clinic of Braunschweig

From the Surgery Clinic of Braunschweig (BS) 28 stents were analysed by SSCP fingerprinting, comprising 27 biliary stents and 1 pancreatic stent. For biliary stents each a fingerprint for the stent end proximal and distal to the liver was generated to check for differences in community structures along the stent. Moreover, two stents showed a biofilm visible by eye on the exterior of the proximal liver end, which was analysed separately using SSCP fingerprinting. The stents originated from 19 patients, and the sample set comprised twice two stents from one patient, twice three stents from one patient and once four stents from one patient. All stents that were originating from the same patient were inserted consecutively, being replaced due to clogging, dislocation or intended stent exchange, e.g. for recovery of histology samples.

Figures 3.2a and 3.2b show the 57 fingerprints generated from stents from BS. The mean number of phylogenetic groups identified to be present per stent was 5.6 with a minimum of 1 and a maximum of 10 (see species richness table appendix A1). As expected, the mean number of phylogenetic groups was found to be higher compared to results of culture-dependent studies carried out on biliary stents previously, which only observed 2-3 different isolates per stent (Di Rosa *et al.*, 1999). Bands for which an assignment to a phylogenetic group was possible are labelled with the SSCP phylotype number. The respective results are summarised in the form of absence/presence as shown in table 3.2. As evident from figures 3.2a and 3.2b, fingerprints generated from different positions of each stent (proximal and distal to the liver) are much more similar than fingerprints from different stents. This could be due to major inter-individual differences and differing inoculation conditions which dilute out the local differences at different regions of the stents. The similarity of fingerprints generated from the same positions is analysed in detail in chapter 3.3.5. In addition to phylotypes from the domain bacteria, human DNA sequences were detected in 4 stents from 3 patients. The amplified fragment has a length of 685 nucleotides and is homologous to a region on human chromosome 9. Further to this, eucaryotic sequences (about 550 bp) were identified in 2 stents of 2 patients showing a high similarity to *Candida* sp. 18S rDNA sequences. This can be explained by only 1 mismatch per Com primer with the 18S rDNA sequence of e.g. *Candida albicans*. Accordingly, (Peters *et al.*, 2000) observed amplification of eucaryotic sequences in their study, where yeast sequences have been amplified unintentionally from compost samples using Com primers.

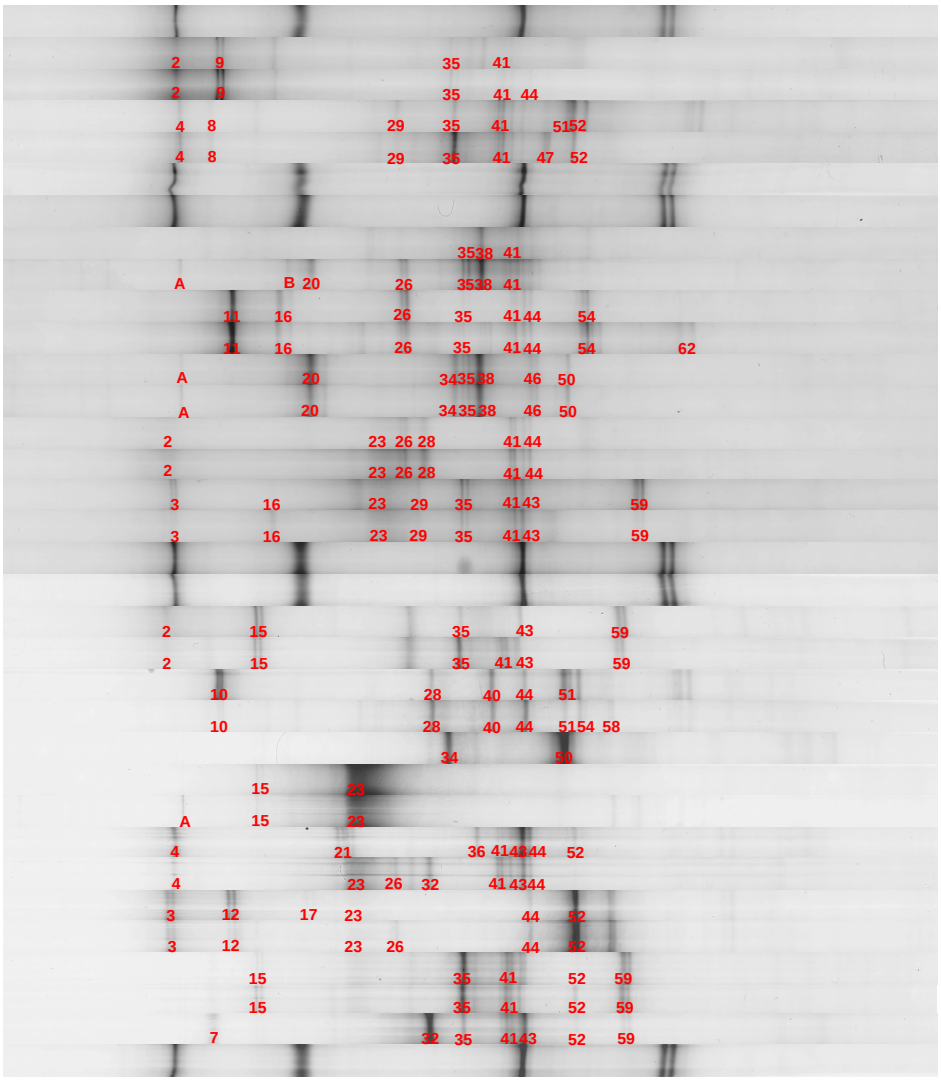

Figure 3.2a: SSCP fingerprints generated from stents from the Surgery Clinic of Braunschweig. 100 ng ssDNA was loaded per lane. All are biliary stents, except if labelled as pancreatic stent. Bands for which phylogenetic information could be retrieved and that contributed to the absence/presence table are marked with the corresponding SSCP phylotype number. Abbreviations: L: side proximal to the liver, L ex: side proximal to the liver exterior, D: side distal to the liver. The marker lane consists of a mixture of single stranded fragments generated from the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

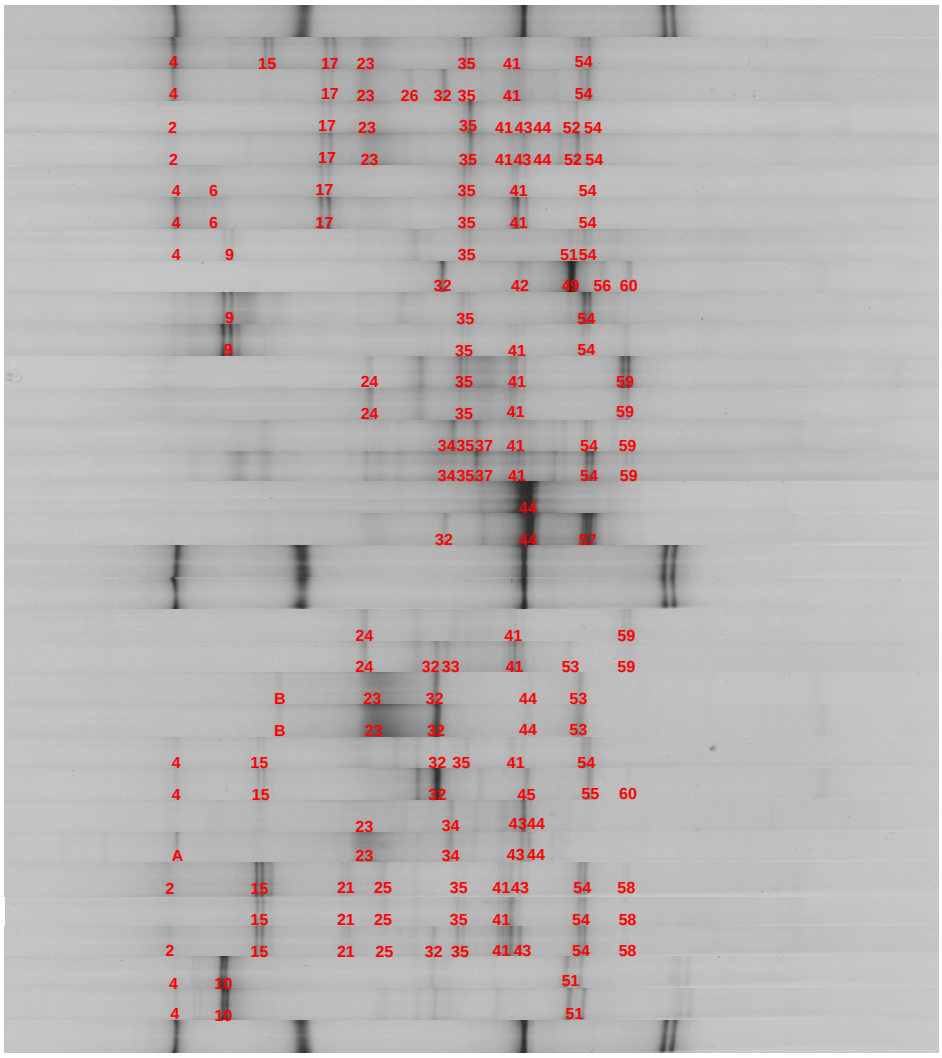

Figure 3.2b: SSCP fingerprints generated from stents from the Surgery Clinic of Braunschweig. 100 ng ssDNA was loaded per lane. All are biliary stents, except if labelled as pancreatic stent. Bands for which phylogenetic information could be retrieved and that contributed to the absence/presence table are marked with the corresponding SSCP phylotype number. Abbreviations: L: side proximal to the liver, L ex: side proximal to the liver exterior, D: side distal to the liver. The marker lane consists of a mixture of single stranded fragments generated from the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

Table 3.2: Occurrence of different phylotypes of the domain bacteria on SSCP fingerprints of biliary stent samples from Surgery Clinic of Braunschweig.

SSCP phylotype number and name	Number of patients		Number of stents		Number of samples					
					Proximal to the liver		Distal to the liver		Pancreatic stent	
total	**19**	**%**	**28**	**%**	**27**	**%**	**27**	**%**	**1**	**%**
41: *Veillonella* sp.	13	68.4	18	64.3	16	59.3	17	63.0	0	0
35: *Streptococcus anginosus*	13	68.4	17	60.7	17	63.0	15	55.6	0	0
23: *Escherichia coli*	9	47.4	9	32.1	8	29.6	9	33.3	0	0
44: *Enterococcus faecium/durans*	9	47.4	10	35.7	9	33.3	10	37.0	0	0
32: *Lactobacillus gasseri*	8	42.1	9	32.1	2	7.4	9	33.3	0	0
54: *Fusobacterium nucleatum* (B)	7	36.8	10	35.7	9	33.3	8	29.6	0	0
4: *Klebsiella oxytoca*	6	31.6	7	25.0	7	25.9	6	22.2	0	0
26: *Bifidobacterium* sp.	6	31.6	6	21.4	2	7.4	6	22.2	0	0
43: *Enterococcus faecalis*	6	31.6	7	25.0	6	22.2	7	25.9	0	0
2: *Klebsiella pneumoniae/ Enterobacter cloacae* subsp. *dissolvens*	5	26.3	5	17.9	5	18.5	5	18.5	0	0
15: *Clostridium perfringens*	5	26.3	6	21.4	6	22.2	4	14.8	0	0
52: *Enterococcus avium*	5	26.3	5	17.9	5	18.5	4	14.8	0	0
34: *Streptococcus oralis/mitis*	4	21.1	4	14.3	3	11.1	3	11.1	1	100
17: *Bacteroides thetaiotaomicron*	3	15.8	4	14.3	4	14.8	3	11.1	0	0
51: *Finegoldia magna*	3	15.8	4	14.3	4	14.8	2	7.4	0	0
59: *Fusobacterium naviforme*	3	15.8	6	21.4	6	22.2	6	22.2	0	0
3: *Enterobacter aerogenes/Pantoea dissolvens*	2	10.5	2	7.1	2	7.4	2	7.4	0	0
9: *Bacteroides fragilis*	2	10.5	3	10.7	3	11.1	2	7.4	0	0
16: *Raoultella planticola*	2	10.5	2	7.1	2	7.4	2	7.4	0	0
21: *Proteus vulgaris*	2	10.5	2	7.1	2	7.4	1	3.7	0	0
29: *Citrobacter freundii*	2	10.5	2	7.1	2	7.4	2	7.4	0	0
50: *Granulicatella adiacens/Abiotrophia paradiacens*	2	10.5	2	7.1	1	3.7	1	3.7	1	100
53: *Lactobacillus mucosae*	2	10.5	2	7.1	1	3.7	2	7.4	0	0
58: *Campylobacter gracilis*	2	10.5	2	7.1	1	3.7	2	7.4	0	0
60: *Lactobacillus salivarius*	2	10.5	2	7.1	0	0	2	7.4	0	0
6: *Bacteroides vulgatus*	1	5.3	1	3.6	1	3.7	1	3.7	0	0
8: *Clostridium ramosus*	1	5.3	1	3.6	1	3.7	1	3.7	0	0
7: *Campylobacter concisus*	1	5.3	1	3.6	0	0	1	3.7	0	0
10: *Synergistes* sp.	1	5.3	2	7.1	2	7.4	2	7.4	0	0
11: *Hafnia alvei/ Obesumbacterium proteus*	1	5.3	1	3.6	1	3.7	1	3.7	0	0
12: *Bacteroides ovatus*	1	5.3	1	3.6	1	3.7	1	3.7	0	0
20: *Rothia mucilaginosus*	1	5.3	2	7.1	1	3.7	2	7.4	0	0
24: *Leptotrichia* sp.	1	5.3	2	7.1	2	7.4	2	7.4	0	0
25: *Prevotella* sp.	1	5.3	1	3.6	1	3.7	1	3.7	0	0
28: *Clostridium* sp. (B)	1	5.3	2	7.1	2	7.4	2	7.4	0	0
33: *Streptococcus sobrinus*	1	5.3	1	3.6	0	0	1	3.7	0	0
36: *Streptococcus parasanguinis*	1	5.3	1	3.6	1	3.7	0	0	0	0
37: *Streptococcus* sp.	1	5.3	1	3.6	1	3.7	1	3.7	0	0
38: *Streptococcus salivarius/thermophilus*	1	5.3	2	7.1	2	7.4	2	7.4	0	0
40: *Enterococcus casseliflavus*	1	5.3	1	3.6	1	3.7	1	3.7	0	0
42: *Lactobacillus delbrueckii* subsp. *lactis*	1	5.3	1	3.6	0	0	1	3.7	0	0
45: *Lactobacillus fermentum*	1	5.3	1	3.6	0	0	1	3.7	0	0
46: *Gemella* sp.	1	5.3	1	3.6	1	3.7	1	3.7	0	0
47: *Haemophilus* sp.	1	5.3	1	3.6	0	0	1	3.7	0	0
49: *Lactobacillus*	1	5.3	1	3.6	0	0	1	3.7	0	0

amylovoris/ultunensis										
55: *Lactobacillus casei/rhamnosus*	1	5.3	1	3.6	0	0	1	3.7	0	0
56: *Lactobacillus reuteri/frumenti*	1	5.3	1	3.6	0	0	1	3.7	0	0
57: *Pediococcus pentosaceus*	1	5.3	1	3.6	0	0	1	3.7	0	0
62: *Providencia rettgeri*	1	5.3	1	3.6	0	0	1	3.7	0	0

Veillonella sp. and *Streptococcus anginosus* are the two most abundant SSCP phylotypes in stents from BS and occur in nearly 70% of all patients. *Escherichia coli* and the phylotype *Enterococcus faecium/durans* are found in nearly 50% of all patients. *Lactobacillus gasseri* and *Fusobacterium nucleatum* occur in about 40% of all patients, whereas *Klebsiella oxytoca*, *Bifidobacterium* sp. and *Enterococcus faecalis* are observed in about 30% of all patients. The above-named phylotypes are all regarded as typical members of the normal duodenal microbiota. Remarkable is the high occurrence of *Veillonella* sp., which is thought to be present in smaller numbers in the duodenum, with the duodenal microbiota being dominated by acid-tolerant species like Streptococci and Lactobacilli (Wilson, 2005).

3.3.3 Medical Clinic of Salzgitter-Lebenstedt

From the Medical Clinic of Salzgitter-Lebenstedt (SZ) 36 stents have been analysed by SSCP fingerprinting, comprising 32 biliary stents and 4 pancreatic stents. All stents were originating from 22 patients, with 6 times 2 stents and 4 times 3 stents inserted simultaneously in the same patient. For both biliary and pancreatic stents a fingerprint was generated for both stent sides. Furthermore, 4 biliary stents showing an exterior biofilm proximal to the liver were analysed separately.

SSCP fingerprints of stents from SZ are presented in figures 3.3a and 3.3b. The mean number of phylogenetic groups identified to be present per stent was 6.3 with a minimum of 2 and a maximum of 11 (see species richness table appendix A2), similar to the diversity observed for stents obtained from BS. The absence/presence data are shown in table 3.3. As with the stents from BS, fingerprints generated for different positions of each stent (proximal and distal to the liver) are much more similar than fingerprints of different stents (see figures 3.3a and 3.3b), thus, pointing to dominating inter-individual differences between the patients. Eucaryotic sequences were also detected on fingerprints from samples of SZ, namely human sequences in 10 stents of 9 patients and yeast sequences in 8 stents of 7 patients (*Candida* and *Saccharomyces* sp.).

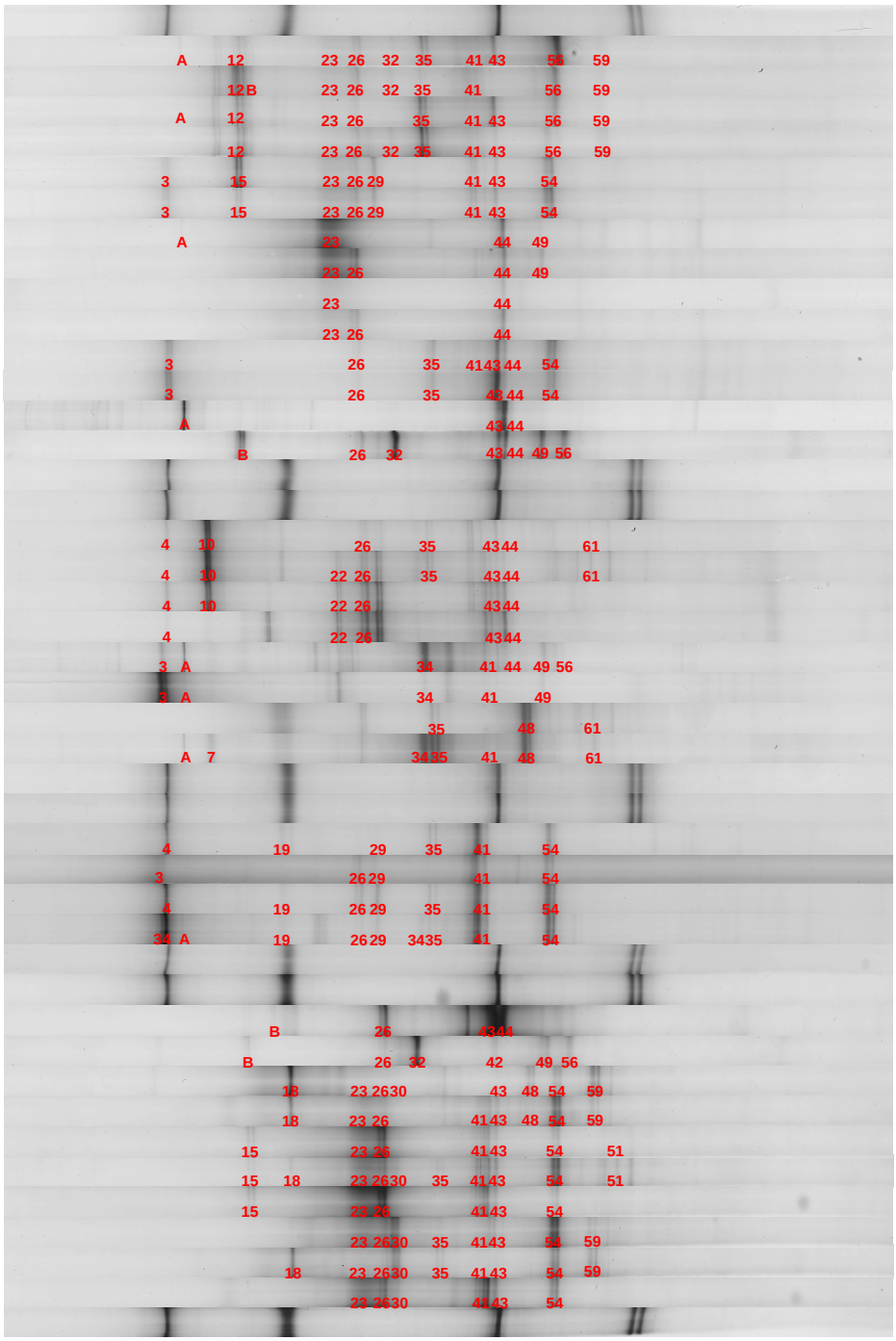

Figure 3.3a: SSCP fingerprints generated from stents of the Medical Clinic of Salzgitter. 100 ng ssDNA was loaded per lane. All are biliary stents, except if labelled as pancreatic stent. Bands for which phylogenetic information could be retrieved and that contributed to the absence/presence table are marked with the corresponding SSCP phylotype number. Abbreviations: L: side proximal to the liver, L ex: side proximal to the liver exterior, D: side distal to the liver, P: side proximal to the pancreas. The marker lane consists of a mixture of single stranded fragments generated from the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

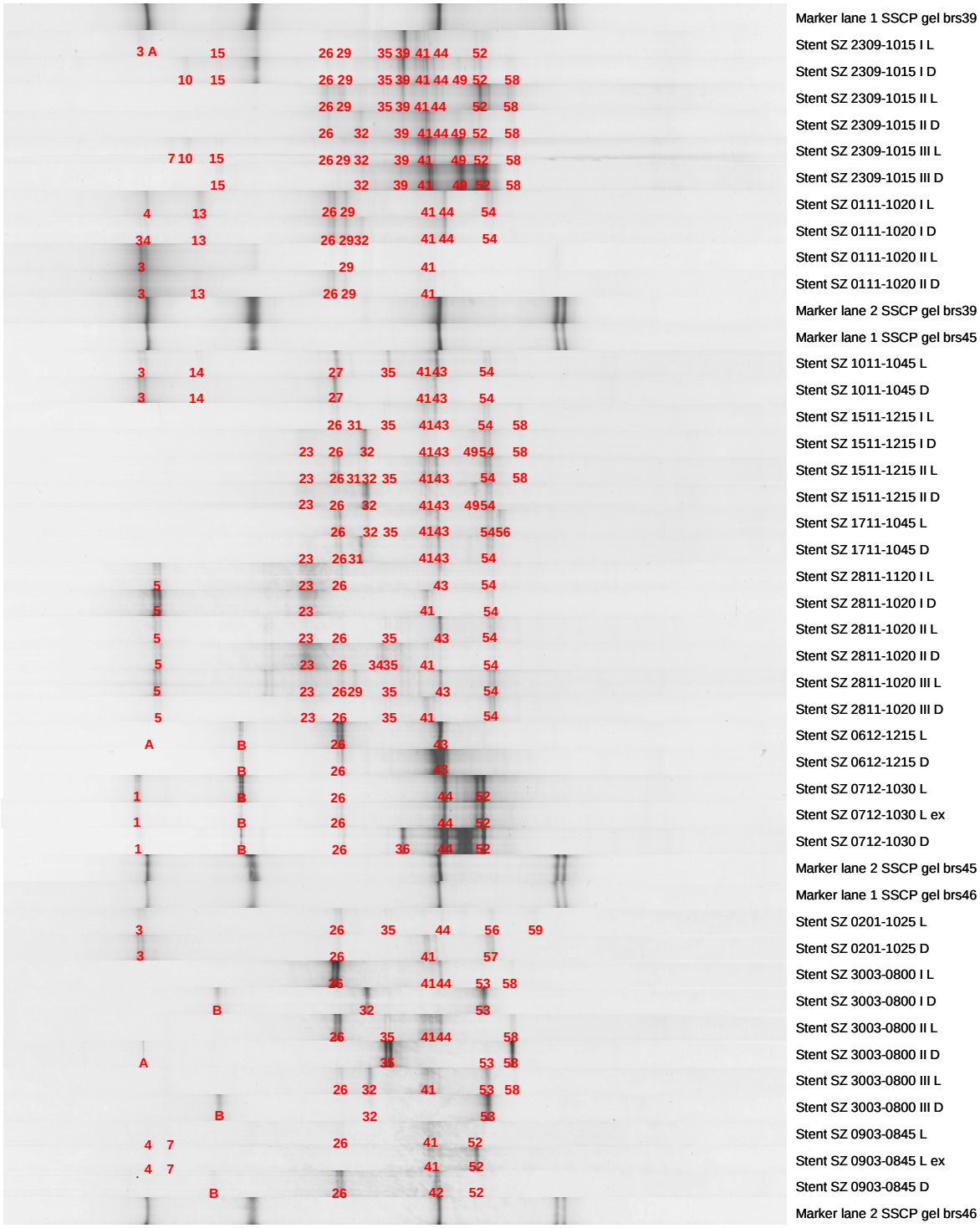

Figure 3.3b: SSCP fingerprints generated from stents of the Medical Clinic of Salzgitter. 100 ng ssDNA was loaded per lane. All are biliary stents, except if labelled as pancreatic stent. Bands for which phylogenetic information could be retrieved and that contributed to the absence/presence table are marked with the corresponding SSCP phylotype number. Abbreviations: L: side proximal to the liver, L ex: side proximal to the liver exterior, D: side distal to the liver, P: side proximal to the pancreas. The marker lane consists of a mixture of single stranded fragments generated from the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

Table 3.3: Occurrence of different phylogenetic groups of the domain bacteria on SSCP fingerprints of biliary stent samples from Medical Clinic of Salzgitter.

SSCP phylotype number and name	Number of patients		Number of stents		Number of samples					
					Proximal to the liver		Distal to the liver		Pancreatic stents	
total	**22**	**in %**	**36**	**%**	**32**	**%**	**32**	**%**	**4**	**%**
26: *Bifidobacterium* sp.	19	86.4	33	91.7	27	84.4	26	81.3	2	50
41: *Veillonella* sp.	16	72.7	28	77.8	21	65.6	20	62.5	3	75
35: *Streptococcus anginosus*	13	59.1	19	52.8	17	53.1	9	28.1	1	25
43: *Enterococcus faecalis*	12	54.5	19	52.8	18	56.3	13	40.6	1	25
44: *Enterococcus faecium/durans*	11	50.0	15	41.7	13	40.6	9	28.1	2	50
54: *Fusobacterium nucleatum* (B)	9	40.9	15	41.7	14	43.8	14	43.8	1	25
32: *Lactobacillus gasseri*	8	36.4	12	33.3	5	15.6	11	34.4	0	0
3: *Enterobacter aerogenes*/*Pantoea agglomerans*	8	36.4	10	27.8	6	18.8	8	25.0	1	25
23: *Escherichia coli*	7	31.8	14	38.9	10	31.3	12	37.5	2	50
49: *Lactobacillus amylovoris/ultunensis*	6	27.3	9	25.0	1	3.1	7	21.9	2	50
56: *Lactobacillus reuteri/frumenti*	6	27.3	7	19.4	4	12.5	4	12.5	1	25
29: *Citrobacter freundii*	5	22.7	9	25.0	9	28.1	6	18.8	0	0
4: *Klebsiella oxytoca*	4	18.2	6	16.7	6	18.8	5	15.6	0	0
34: *Streptococcus oralis/mitis*	4	18.2	4	11.1	0	0	2	6.3	2	50
58: *Campylobacter gracilis*	3	13.6	8	22.2	7	21.9	5	15.6	0	0
52: *Enterococcus avium*	3	13.6	5	13.9	5	15.6	5	15.6	0	0
59: Fusobacterium naviforme	3	13.6	5	13.9	4	12.5	2	6.3	1	25
15: *Clostridium perfringens*	3	13.6	4	11.1	4	12.5	4	12.5	0	0
7: *Campylobacter concisus*	3	13.6	3	8.3	2	6.3	0	0	1	25
39: *Streptococcus alactolyticus/gallolyticus* subsp. *macedonicus*	2	9.1	4	11.1	3	9.4	4	12.5	0	0
10: *Synergistes* sp.	2	9.1	4	11.1	3	9.4	2	6.3	0	0
31: *Streptococcus bovis*	2	9.1	3	8.3	2	6.3	1	3.1	0	0
61: *Peptostreptococcus micros*	2	9.1	2	5.6	1	3.1	1	3.1	1	25
42: *Lactobacillus delbrueckii* subsp. *lactis*	2	9.1	2	5.6	0	0	2	6.3	0	0
48: *Fusobacterium nucleatum* (A)	2	9.1	2	5.6	0	0	0	0	2	50
5: *Bacteroides dorei*	1	4.5	3	8.3	3	9.4	3	9.4	0	0
53: *Lactobacillus mucosae*	1	4.5	3	8.3	2	6.3	3	9.4	0	0
12: *Bacteroides ovatus*	1	4.5	2	5.6	2	6.3	2	6.3	0	0
19: *Selenomonas* sp.	1	4.5	2	5.6	2	6.3	1	3.1	0	0
13: *Bilophila wadsworthia*	1	4.5	2	5.6	1	3.1	2	6.3	0	0
22: *Clostridium* sp. (A)	1	4.5	2	5.6	1	3.1	2	6.3	0	0
30: *Prevotella oralis*	1	4.5	2	5.6	1	3.1	1	3.1	1	25
1: *Propionibacterium acidipropionici*	1	4.5	1	2.8	1	3.1	1	3.1	0	0
14: *Aeromonas hydrophila*	1	4.5	1	2.8	1	3.1	1	3.1	0	0
27: *Morganella morganii*	1	4.5	1	2.8	1	3.1	1	3.1	0	0
51: *Finegoldia magna*	1	4.5	1	2.8	1	3.1	0	0	0	0
57: *Pediococcus pentosaceus*	1	4.5	1	2.8	0	0	1	3.1	0	0
18: *Porphyromonas gingivalis*	1	4.5	1	2.8	0	0	0	0	1	25

In contrast to stents from BS, however, *Bifidobacterium* sp. was the most abundant SSCP phylotype observed in stents from SZ (approximately 85% of all patients). *Veillonella* sp., *Streptococcus anginosus*, Enterococci and *Fusobacterium nucleatum* were observed at a level similar to that in stents from BS. As for BS, typical members of the duodenal microbiota are found to be dominant in stent community composition, although the high abundance of *Bifidobacterium* sp. and *Veillonella* sp. is inconsistent with abundances observed in the normal duodenal microbiota.

3.3.4 Comparison of the bacterial community composition of stents from both hospitals

In order to elucidate communalities in biliary stent community composition, but also possible differences that arise from different stent management routines and patient treatments, results of SSCP fingerprinting of stents obtained from BS and SZ are compared.

3.3.4.1 Combined abundance of phylotypes in biliary stents evaluated at the family level

In order to allow a clearer comparison of phylotypes detected in biliary stents between hospitals, the presence of phylogenetic families observed across all fingerprints from each of the hospitals was combined and their occurrence at either the proximal or distal liver ends was summed. As shown in figure 3.4, representatives of overall 16 bacterial families could be detected in stents from BS with members of the Enterobacteriaceae, Streptococcaceae and Enterococcaceae each accounting for 13-18% of all phylotypes for all stents. Lactobacillaceae, Acidaminococcaceae and Fusobacteriaceae were quite abundant as members of biliary stent biofilms (accounting for more than 10% of phylotypes for all stents of BS). In addition, members of the Bacteroidaceae, Clostridiaceae, Bifidobacteriaceae and Peptostreptococcaceae were less frequently observed (observed in more than 2% of phylotypes for all stents of BS). A comparison of community members observed at the different regions of stents from BS showed Lactobacillaceae to be more frequently observed on the distal liver side compared to the proximal liver side (12 compared to 2% of phylotypes, respectively). For all of the other phylogenetic groups, there are no further articulate differences between the different regions of the stents. In stents from SZ overall 17 bacterial families could be

detected (figure 3.4). The most abundant phylogenetic groups at the family level from SZ stents are Enterobacteriaceae, Enterococcaceae, Lactobacillaceae, Bifidobacteriaceae, Streptococcaceae and Acidaminococcaceae (accounting for more than 10% of phylotypes for all stents). Approximately 10 additional minor groups are observed with Fusobacteriaceae and Campylobacteraceae being the most prominent among them (accounting for more than 2% of phylotypes for all stents). When comparing the community composition at both regions of stents from SZ the most obvious difference is, like observed for stents from BS, the higher occurrence of Lactobacillaceae at the distal liver end (14 compared to 6% of phylotypes). The most striking difference in the combined abundances of phylogenetic families in stents from BS and SZ is the relatively high abundance of Bifidobacteriaceae in stents from SZ (12 compared to 3% of phylotypes for all stents from SZ and BS, respectively). On the other hand, members of the families of Clostridiaceae and Bacteroidaceae are more abundant in samples from BS (5% compared to 2% of phylotypes for all stents from BS and SZ, respectively).

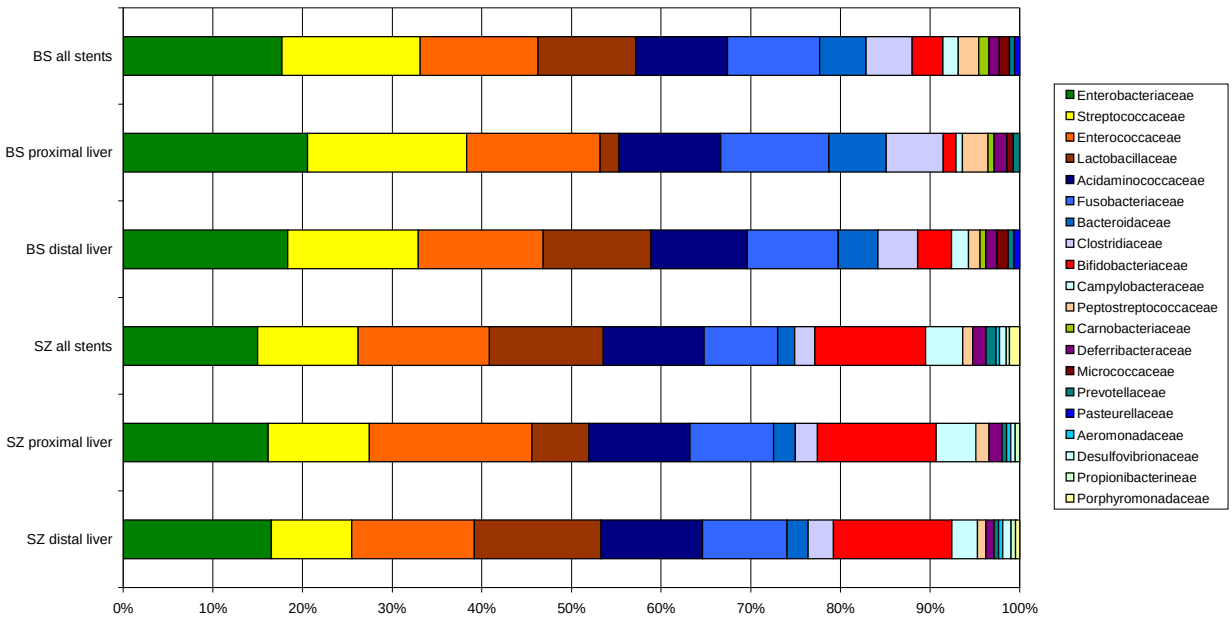

Figure 3.4: Families of the domain bacteria observed on SSCP fingerprints of stents from Surgery Clinic of Braunschweig and Medical Clinic of Salzgitter. The combined abundance of bacterial families in all stents from each hospital and the combined abundance of bacterial families at the proximal and distal liver end of stents from each hospital are shown separately.

3.3.4.2 Combined abundance of phylotypes in biliary stents evaluated at the phylum level

In order to allow a comparison with the microbiota observed in a culture-independent study of the jejunum (Wang *et al.*, 2005), which is spatially the closest habitat to the duodenum that has been studied up to now using culture-independent methods, the combined abundances of phylotypes at the phylum level as observed for stents of each hospital, as well as the community structure of the human jejunum at the phylum level (Wang *et al.*, 2005) are summarised in figure 3.5. The most apparent difference between the two hospitals is the higher abundance of Actinobacteria in samples from SZ, while the members of the Bacteroidetes, Firmicutes and Fusobacteria are slightly reduced in comparison with BS. Overall, at this level of phylogenetic hierarchy SSCP fingerprinting resulted in very similar findings for both hospitals. A higher abundance of Firmicutes is observed in the jejunum (80 compared to approximately 60% in the stents), while Proteobacteria are observed less frequently (13 compared to 20% for the stents). Bacteroidetes are observed in minor amounts in both habitats. Fusobacteria and Actinobacteria seem to be more abundant in the stent communities compared to the jejunum microbiota. Overall, taking into account the change of the microbiota along the intestinal tract, microbial community structure observed over the mean of all stents can be regarded as similar to the normal duodenal microbiota.

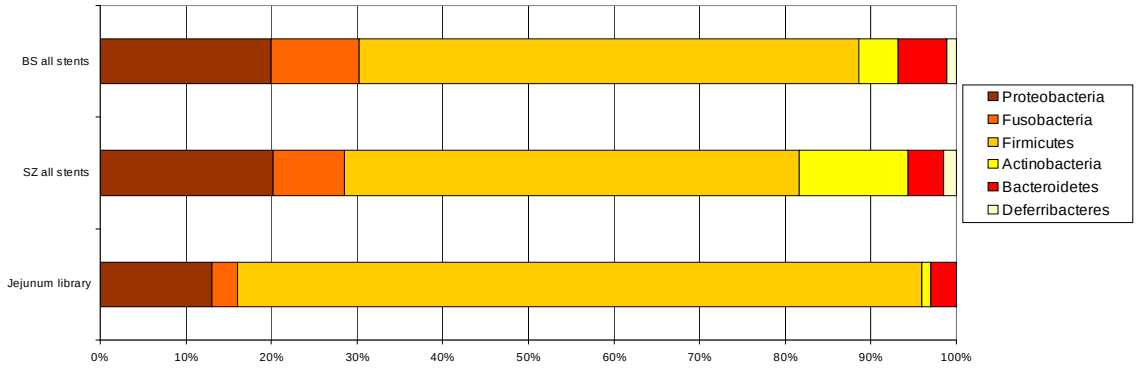

Figure 3.5: Combined abundance of phyla of the domain bacteria observed on SSCP fingerprints of stents from Surgery Clinic of Braunschweig and Medical Clinic of Salzgitter, and phyla of the domain bacteria observed in the 16S rDNA library of the human jejunum (Wang *et al.*, 2005).

3.3.4.3 Multivariate statistical analysis of fingerprints from both hospitals

To further analyse the similarities and differences of microbial communities from both hospitals non-metric multivariate statistical analysis was performed on SSCP fingerprints, using PRIMER v6 (Clarke, 1993). After calculation of Bray-Curtis similarity matrices, multi-dimensional scaling plots (MDS) were generated and groups of fingerprints tested by analysis of similarity (ANOSIM). Figure 3.6 shows the non-metric 2D MDS plot of all fingerprints from BS and SZ. The stress value of 0.2, being a measure of distortion of the plot, is acceptable considering that 133 fingerprints are positioned in the plot, thus giving a useful 2 dimensional picture. No distinct visual clustering of samples from the 2 different hospitals is observed in the plot. However, there is a slight divergence of samples from BS (green dots) to the left and SZ (blue dots) to the right side of the plot. This indicates that the bacterial communities of stents may be faintly different between the hospitals as shown above by comparing composition at the family level, which indicated a higher occurrence of Bifidobacteriaceae in communities of SZ.

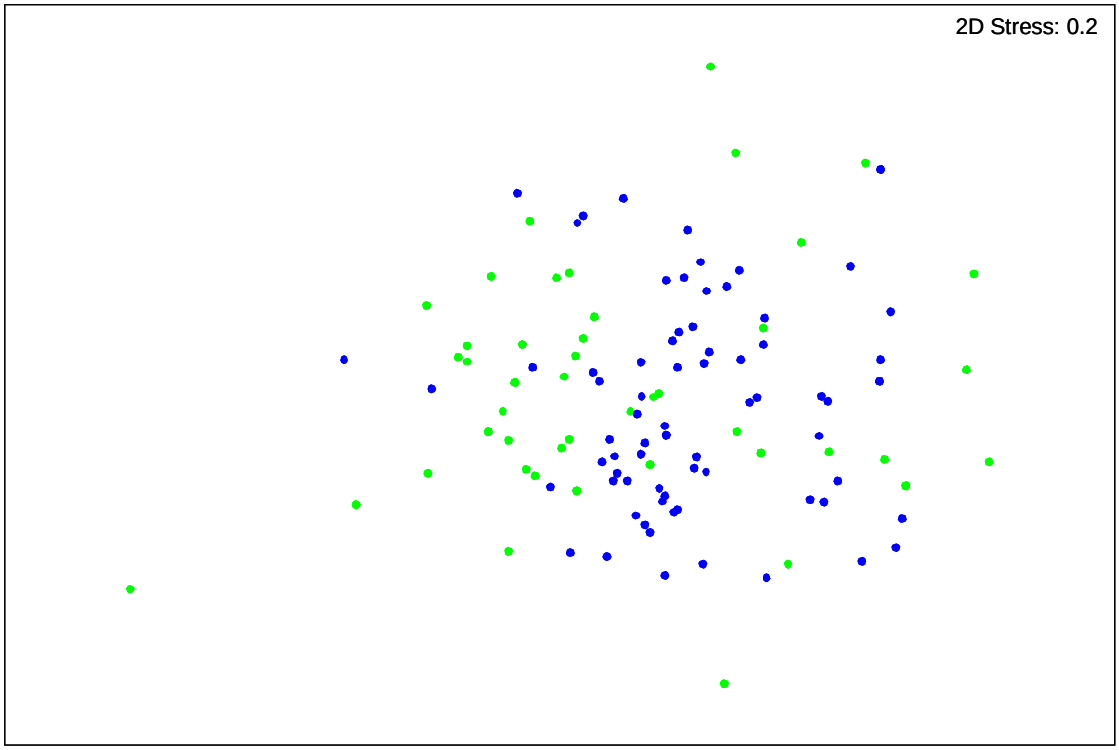

Figure 3.6: Multidimensional scaling plot based on Bray-Curtis similarity of PCR-SSCP fingerprint data from Surgery Clinic of Braunschweig (green) and Medical Clinic of Salzgitter (blue) consisting of presence/absence of phylogenetic groups. The stress value is indicated.

The slight difference between microbial communities observed in stents from both hospitals was confirmed by a one-way analysis of similarity (ANOSIM), which demonstrates a significant difference between samples from each hospital ($p<0.001$). However, the global R-value is very low ($R=0.181$), thus, while microbial community structures are statistically different they are still barely separable.

3.3.5 Comparing the communities at different positions of the stents

As multivariate statistical analysis has revealed statistically significant differences between samples from the two hospitals, this analysis was performed to elucidate whether statistically significant differences are as well evident between communities established at different parts of the analysed stents, the end proximal and distal to the liver, the exterior of the end proximal to the liver and the end proximal to the pancreas of pancreatic stents (figure 3.7). The MDS plot is identical to the one presented in figure 3.6, but labels the samples according to the position of the stents at which DNA was extracted.

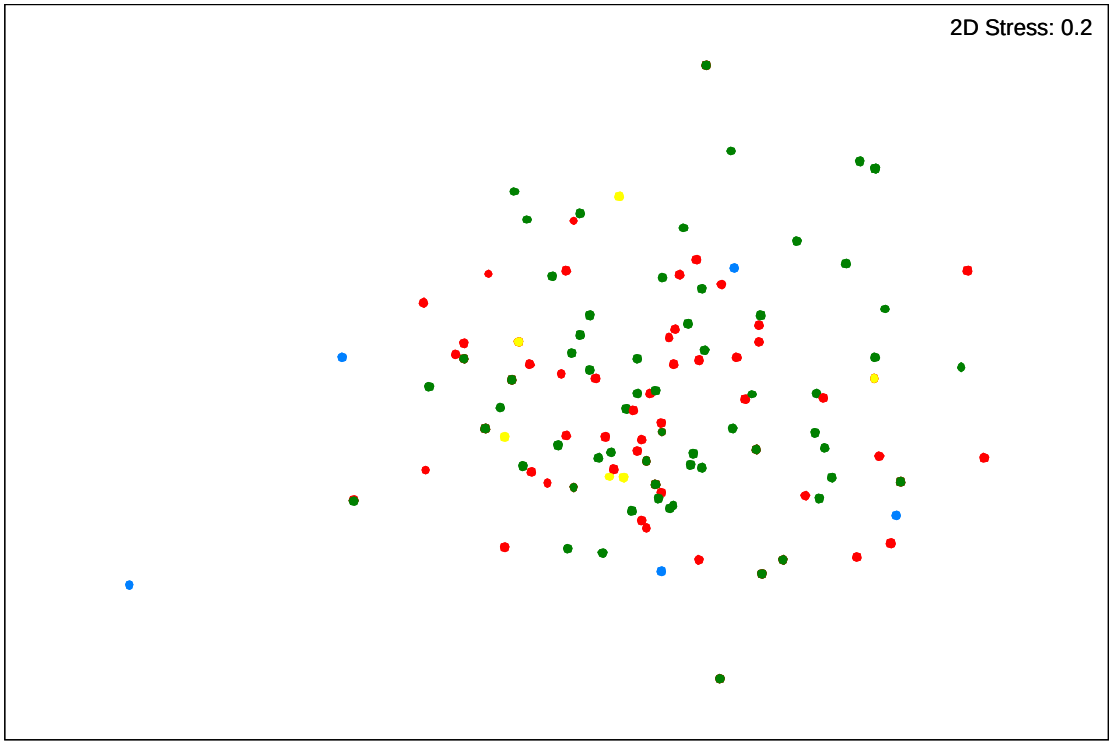

Figure 3.7: Multidimensional scaling plot based on Bray-Curtis similarity of PCR-SSCP fingerprint data from Surgery Clinic of Braunschweig and Medical Clinic of Salzgitter consisting of presence/absence of phylogenetic groups. Position proximal to the liver (red), position distal to the liver (green), position proximal to the liver exterior (yellow) and position proximal to the pancreas (blue). The stress value is indicated.

No evident clustering and thus no directly evident general differences in community structures between the different locations of the stent biofilms are observed. However, the bacterial community structures between individual stent biofilms having been located proximal to the pancreas (blue dots) were highly variable, as indicated by the distance in the MDS plot. This indicates that the species composition is more different within this group of samples. The same analysis was performed for the data set of fingerprints from each hospital separately, however, no further trend of separation could be observed.

To further test if there are significant differences between communities at the different stent locations, which are not directly visible in the MDS plot, a one-way ANOSIM analysis was performed. As this revealed significant differences between community structures from each sampling position ($p<0.028$), however, with a very low global R-value (0.043), pairwise comparisons were carried out for microbial community structures of all sampling positions as summarised in table 3.4. Only the comparisons of proximal liver/proximal pancreas, distal liver/proximal pancreas and proximal liver exterior/proximal pancreas show a significant difference between bacterial community structures. That is, the bacterial community structure of the proximal and distal liver position, the proximal liver and exterior of proximal liver position and the distal and proximal liver exterior were similar (statistically inseparable $p>0.05$). It makes sense that communities established in the pancreatic stents are different from those of the biliary stents, constituting a different habitat.

Table 3.4: Pairwise comparisons of the one-way ANOSIM of microbial communities at different sampling positions. Significant comparisons ($p<0.05$) are presented in bold.

Pairwise comparison	R statistic	Significance level (p<)
Proximal liver/distal liver	0.01	0.187
Proximal liver/proximal liver exterior	<0	0.583
Proximal liver/proximal pancreas	**0.374**	**0.004**
Distal liver/proximal liver exterior	<0	0.531
Distal liver/proximal pancreas	**0.271**	**0.020**
Proximal liver exterior/proximal pancreas	**0.276**	**0.043**

3.3.6 Host influence on microbial community structure

In order to analyse the influence of inter-individual differences between patients, cases where several stents were removed from one patient were analysed in more detail. This is first shown for samples from BS, where stents from each patient are implanted consecutively over time and second for samples from SZ, where stents from each patient are implanted simultaneously (see sections 3.3.1. and 3.3.2 for more detail).

3.3.6.1 Consecutively implanted biliary stents of the same patients

Figure 3.8 shows the 2D MDS plot for all SSCP fingerprints from patients of BS from whom several stents were obtained (patients A-E). In total, 29 fingerprints are plotted and the stress of the plot is 0.09, corresponding to a good ordination. Microbial communities observed in stents from the same patients are obviously similar to each other, with microbial communities obtained from patient D showing the highest within-patient difference.

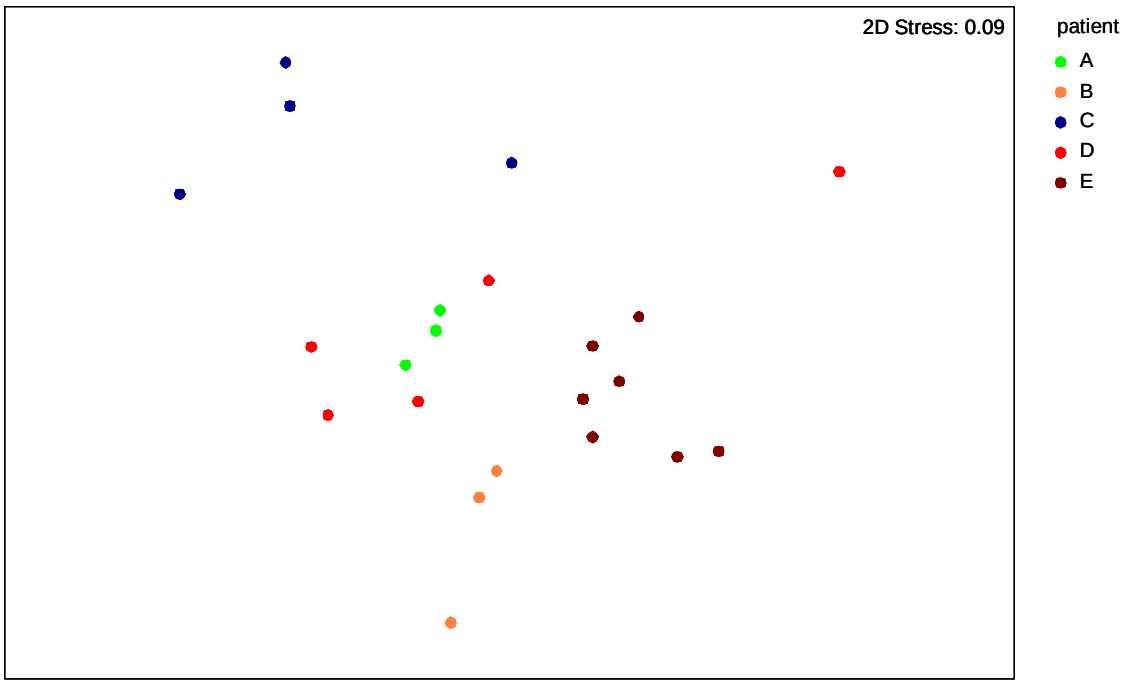

Figure 3.8: Multidimensional scaling plot based on Bray-Curtis similarity of PCR-SSCP fingerprint data consisting of presence/absence of phylogenetic groups from patients from Surgery Clinic Braunschweig of whom several samples were obtained (patients A-E). The stress value is indicated.

A one-way ANOSIM confirmed that there are statistically significant differences between each group of samples from the same patient. The global R-value obtained was 0.652 at a significance level of $p<0.001$. Thus, the groups of stents from each patient can be regarded as clearly different, although there is some overlapping between the respective communities. Table 3.5 presents the pairwise comparisons of the one-way ANOSIM. Except for the comparison of biofilm communities from patient A and D ($R=0.113$, $p<0.219$) all comparisons are statistically significant (significance level of $p<0.05$). The R-values of these comparisons are in the range of 0.4 – 1, indicating a clear separation of groups, although some overlapping is indicated. This indicates that for all patients (except A and D) bacterial community structure is different. Patient's A stents and patient's D stents clearly share a similar bacterial community composition.

Table 3.5: Pairwise comparisons of the one-way ANOSIM of sample groups from the same patients of Surgery Clinic Braunschweig. Significant comparisons ($p<0.05$) are presented in bold.

Pairwise comparison	R statistic	Significance level (p<)
A/B	**0.958**	**0.029**
A/C	**0.595**	**0.014**
A/D	0.113	0.219
A/E	**0.848**	**0.001**
B/C	**0.754**	**0.005**
B/D	**0.46**	**0.010**
B/E	**0.83**	**0.001**
C/D	**0.406**	**0.009**
C/E	**0.882**	**0.001**
D/E	**0.644**	**0.001**

To determine which of the SSCP phylotypes contribute to the similarity and dissimilarity of biofilm microbial structures, similarity percentages analysis (SIMPER) was used, which breaks down the average similarity of intra-group sample pairs and the average dissimilarity of inter-group sample pairs to contributions of each phylotype. Table 3.6 presents the summary of SSCP phylotypes contributing more than 10% to the cumulative similarity or dissimilarity between groups of microbial communities. For dissimilarity analysis, groups of samples from BS were compared in 10 pairwise comparisons, the comparison of patient A and D was excluded since no significant separation of these groups was shown in the ANOSIM. One comparison yielded no phylogenetic group having more than 10% contribution in dissimilarity analysis

(microbial community structures of patient C compared to D). As an example, all microbial communities of patient E had an average similarity of 59%, and *Fusobacterium nucleatum* (B) contributed 35%, *Veillonella* sp. 27%, and *Streptococcus anginosus* 20% to the similarity. All other SSCP phylotypes contributed less than 10% to the overall similarity. For the dissimilarity of microbial communities of patient E and B (average 74%) *Streptococcus salivarius/thermophilus* and *Fusobacterium naviforme* contributed both 14%, all other SSCP phylotypes contributed less than 10%.

Overall of the total of 62 phylogenetic groups detected on PCR-SSCP fingerprints, 13 contributed more than 10% to the similarity within a sample group. Particularly *Streptococcus anginosus* contributed to the similarity in 4 of 5 and *Fusobacterium nucleatum* (B) and *Veillonella* sp. in 2 of 5 analysed groups of microbial communities. For the dissimilarity analysis 6 SSCP phylotypes were identified as most important for dissimilarity between microbial communities of different patients. Interestingly, all of these 6 SSCP phylotypes were also found important in the similarity analysis.

Table 3.6: Results of the similarity and dissimilarity analysis for patients of BS using SIMPER. SSCP phylotypes having a contribution of more than 10% are presented.

SSCP phylotype	Contributing to x out of 5 groups (A – E)	Contributing to x out of 9 comparisons
54: *Fusobacterium nucleatum* (B)	2	4
38: *Streptococcus salivarius/thermophilus*	1	4
59: *Fusobacterium naviforme*	1	4
17: *Bacteroides thetaiotaomicron*	1	3
35: *Streptococcus anginosus*	4	2
4: *Klebsiella oxytoca*	1	2
41: *Veillonella* sp.	2	-
9: *Bacteroides fragilis*	1	-
20: *Rothia mucilaginosus*	1	-
10: *Synergistes* sp.	1	-
51: *Finegoldia magna*	1	-
28: *Clostridium* sp. (B)	1	-
44: *Enterococcus faecium/durans*	1	-

3.3.6.2 Simultaneously implanted biliary stents of the same patients

Microbial communities established in stents simultaneously inserted in one patient from SZ are presented in a 2D MDS plot (figure 3.9). Fifty fingerprints from 10 patients are summarised in this plot with a stress value of 0.18 giving a useful 2-dimensional picture. The groups of fingerprints from the same patient cluster together, as already observed for microbial communities from the same patient of BS. Some overlapping between patients I and L indicates similar microbial communities. Samples from patient O are found to be quite diverse, although this is not indicative for a time trend in this case.

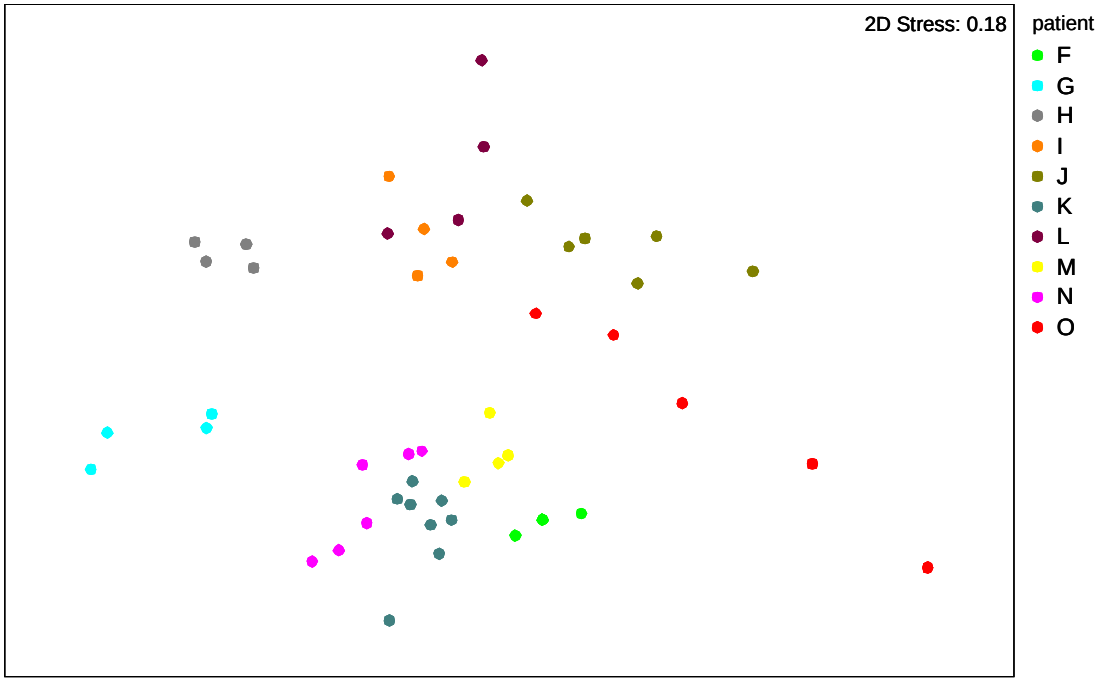

Figure 3.9: Multidimensional scaling plot based on Bray-Curtis similarity of PCR-SSCP fingerprint data consisting of presence/absence of phylogenetic groups from patients from Medical Clinic Salzgitter of whom several samples were obtained (patients F-O). The stress value is indicated.

A one-way ANOSIM was used to test whether the observed differences between groups are of statistical significance. The global R-value obtained was 0.872 at $p<0.001$. Thus, the groups can be regarded as very well separable. In the pairwise comparisons, only samples of patient I and L were not significantly different ($R=0.432$, $p<0.086$). All other 44 pairwise comparisons were significantly different ($p<0.05$) and delivered R-values between 0.6 and 1. In total an R-value of 1 was calculated for 17 of

the 45 pairwise comparisons. This indicates that the bacterial community structure of stents from most patients was different to each other, except for patients I and L, who shared similar community structures.

Additionally a SIMPER was performed for groups of microbial communities from SZ patients. Table 3.7 presents the summary of SSCP phylotypes contributing more than 10% to the cumulative similarity or dissimilarity between groups of microbial communities. For dissimilarity analysis, groups of samples from SZ were compared in 44 pairwise comparisons. The comparison of patient I and L was excluded since no significant separation of these groups was shown in the ANOSIM. Eight comparisons yielded no SSCP phylotype having more than 10% contribution in dissimilarity analysis (F & H, F & I, F & J, F & L, H & J, I & J, J & K, J & N). As an example, microbial communities of patient N showed an average similarity of 76%, with a contribution of more than 10% of *Bacteroides dorei*, *Fusobacterium nucleatum* (B), *Escherichia coli* (all 23%) and *Bifidobacterium* sp. (14%). The dissimilarity analysis between patient N and M (average dissimilarity 43%) demonstrated *Bacteroides dorei* (17% contribution), *Lactobacillus gasseri* (13% contribution) and *Campylobacter gracilis* (13% contribution) to be most important for the dissimilarity between both groups of microbial communities.

Overall of the total of 62 phylogenetic groups detected on PCR-SSCP fingerprints, 20 contributed to more than 10% of the similarity within a sample group. The most important were *Bifidobacterium* sp. contributing in 7 of 10 groups and *Veillonella* sp. in 6 of 10 groups. For the dissimilarity analysis 22 SSCP phylotypes were identified as contributing more than 10% to the dissimilarity between microbial communities of different patients. The most common was *Fusobacterium nucleatum* (B) contributing in 16 comparisons. Interestingly, the SSCP phylotype *Bifidobacterium* sp. that was identified as most important in similarity within groups did not contribute noticeably to the dissimilarity between groups.

Table 3.7: Results of the similarity and dissimilarity analysis for patients of SZ using SIMPER. SSCP phylotypes having a contribution of more than 10% are presented.

SSCP phylotype	Contributing to x out of 10 groups (F – O)	Contributing to x out of 44 comparisons
54: *Fusobacterium nucleatum* (B)	4	16
23: *Escherichia coli*	4	14
43: *Enterococcus faecalis*	3	13
29: *Citrobacter freundii*	2	12
44: *Enterococcus faecium/durans*	2	10
41: *Veillonella* sp.	6	9
5: *Bacteroides dorei*	1	8
53: *Lactobacillus mucosae*	1	7
4: *Klebsiella oxytoca*	1	6
56: *Lactobacillus reuteri/frumenti*	1	5
12: *Bacteroides ovatus*	1	5
58: *Campylobacter gracilis*	2	4
59: *Fusobacterium naviforme*	1	4
32: *Lactobacillus gasseri*	1	4
39: *Streptococcus alactolyticus/gallolyticus* subsp. *macedonicus*	1	4
52: *Enterococcus avium*	1	4
35: *Streptococcus anginosus*	1	2
3: *Enterobacter aerogenes /Pantoea agglomerans*	1	2
30: *Prevotella oralis*	-	2
10: *Synergistes* sp.	-	1
19: *Selenomonas* sp.	-	1
22: Clostridium sp. (A)	-	1
26: *Bifidobacterium* sp.	7	-
13: *Bilophila wadsworthia*	1	-

3.3.6.3 Dispersion analysis

In order to identify if microbial communities of stents having been simultaneously inserted in the same patient were more similar to each other compared to microbial communities of stents having been consecutively inserted, dispersion analysis was used. The lower the dispersion index, the tighter the grouping of samples within a group. Table 3.8 shows that groups of microbial communities established in stents from patients from SZ have considerably lower dispersion indices compared to groups of microbial communities established in stents from patients from BS. Thus, groups of stents that were implanted consecutively show a larger within group heterogeneity compared to the clearly tighter grouping of samples implanted simultaneously. This indicates that the bacterial community structure of stents implanted at the same time is much more similar than when stents are implanted in succession in the same patient. Interestingly, the highest dispersion index for SZ and BS patients is found in the group of microbial communities established in stents from patients O and D, respectively. This corresponds well with the observations made from the MDS plots.

Table 3.8: Dispersion indices of groups of microbial communities established in stents originating from the same patient.

Group [1]	Dispersion index
F	0.140
H	0.527
A	0.630
G	0.660
M	0.684
N	0.721
I	0.739
K	0.754
J	0.889
L	1.038
B	1.147
E	1.213
O	1.375
C	1.451
D	1.487

[1] *A-E*: patients of Surgery Clinic of Braunschweig who had stents implanted consecutively, **F-O**: patients of Medical Clinic of Salzgitter who had stents implanted simultaneously.

3.3.7 Is the biliary stent bacterial community structure influenced by patient characteristics?

Patient characteristics such as patient age, gender, primary disease and time and duration of stent placement were obtained for patients from BS. It would have been desirable to obtain data on more parameters like blood values of inflammatory markers or detailed information on medication of the patients for the complete data set. However, this proved to be rather difficult. Using the available information, we analysed if the stent community structures are influenced by the residence time of the stent in the patient or the age of the patient using MDS bubble plots where residence time or age are indicated by bubble size. The community structures of 28 stents as revealed by 57 fingerprints are placed in the plots shown in figure 3.10. The stress of the MDS representation is 0.15 giving an useful 2-dimensional picture. However, neither of the above mentioned factors seems to have an effect on bacterial community structure of the stents analysed.

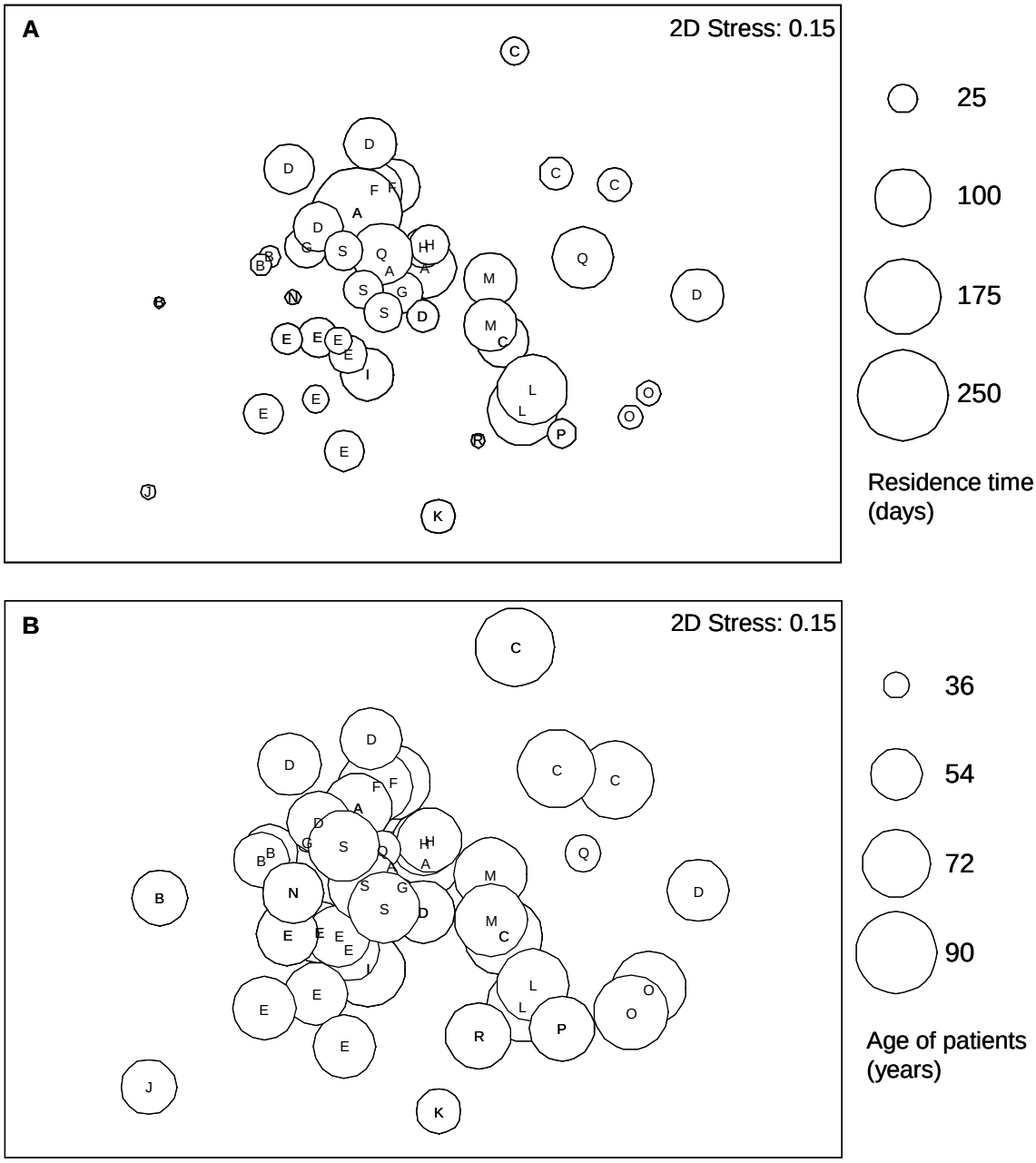

Figure 3.10: 2D MDS bubble plots showing all fingerprints from Surgery Clinic Braunschweig labelled with patient identiy (A-S) and a bubble corresponding in size to the days of implantation of stents (A) or the age of patients (B).

3.3.8 RT-PCR SSCP fingerprints of selected samples

Whereas community structure analysis based on 16S rDNA sequencing is assumed to give an overview on the abundance of the bacterial species present, analyses based on 16S rRNA are assumed to give an overview on active species, which should be rich in ribosomes. However, a continuous fingerprinting of the 16S rRNA present in biliary stent biofilms was not possible, since usually only faint amounts of ribosomal RNA could be extracted, probably due to a low number of ribosomes. This is in accordance with a study of Swidsinski *et al.* (Swidsinski *et al.*, 2005), where biliary stent biofilms were analysed using fluorescence in situ hybridisation (FISH). Very intense conditions had to be applied to gain a signal and even though bacteria resembling structures where observed through autofluorescence and Gram stain in high numbers in completely occluded stents, only poor hybridisations signals where obtained with bacterial probes. Similarly, in this work only in case of a restricted range of samples extraction of sufficient RNA for RT-PCR and following SSCP analysis could be obtained. As an example 16S rRNA RT-PCR SSCP fingerprints of 2 stents from SZ, which have been implanted simultaneously are compared with the 16S rDNA PCR SSCP fingerprints.

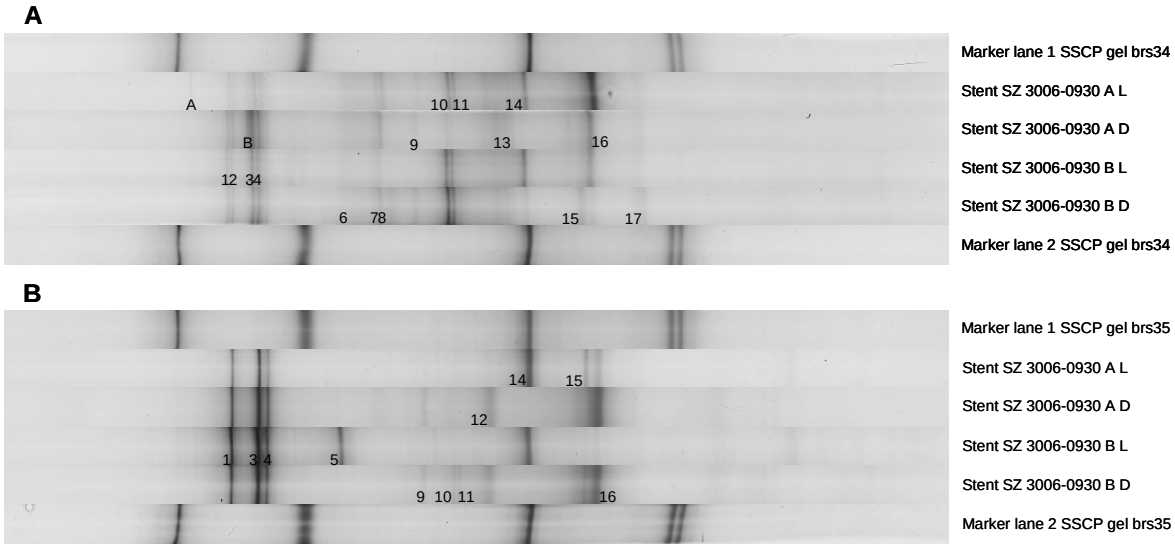

Figure 3.11: PCR-SSCP analysis of 16S rDNA (A) and of 16S rRNA (B) of two stents of the Medical Clinic of Salzgitter. Abbreviations: L: side proximal to the liver, D: side distal to the liver.

Figure 3.11 A presents the 16S rDNA SSCP fingerprints of the proximal and distal liver end of both stents. Several bands could be excised, reamplified and sequenced, and the results of the phylogenetic classification are listed in table 3.9. Besides the generated sequences, band 6 could be identified as *Escherichia coli* and bands 7 and 8 as *Bifidobacterium* sp. by comparison with fingerprints, which were run on the same gel. Furthermore, bands excised at positions A and B yielded sequences with high similarity to human DNA sequences and *Candida* sp., respectively. The 16S rRNA SSCP fingerprints of the proximal and distal liver end of both stents are presented in figure 3.11 B. Excised and reamplified bands are listed with their phylogenetic classification in table 3.9. Furthermore, band 9 could be classified as *Lactobacillus gasseri* and bands 10 and 11 as *Streptococcus anginosus* by comparison of fingerprints, which were run on the same gel.

Table 3.9: Sequences reamplified from SSCP bands of the 16S rDNA and 16S rRNA fingerprints and their phylogenetic closest neighbour according to Sequence match of RDP II.

Sequence identity	Sequence length [1]	Similarity score [2]	Seqmatch score (S_ab) [3]	Phylogenetic closest neighbour	NCBI accession number
1 (DNA & RNA)	367	1.000	1.000	*Bacteroides ovatus*; SDG-Mt85-3Cy	DQ100446
2 (DNA)	367	1.000	1.000	*Bacteroides ovatus*; SDG-Mt85-3Cy	DQ100446
3 & 4 (DNA & RNA)	367	1.000	1.000	*Bacteroides ovatus*; JCM 5824T	AB050108
5 (RNA)	366	1.000	1.000	*Prevotella nanceiensis*; LBN 297	EF405529
6 (DNA*)	370	1.000	1.000	*Escherichia coli*	J01695
7 & 8 (DNA*)	372	1.000	1.000	*Bifidobacterium animalis* subsp. *animalis* (T); DSM 10140	X89513
9 (DNA & RNA*)	371	0.997	0.991	*Lactobacillus gasseri;* ATCC 33323	AF519171
10 & 11 (DNA & RNA*)	370	1.000	1.000	*Streptococcus anginosus;* 1204	AF145240
12 (RNA)	371	0.997	0.986	*Lactobacillus reuteri* (T); DSM 20016 T;	X76328
		0.997	0.986	*Lactobacillus frumenti* (T);	AJ250074

				TMW 1.666;	
13	370	1.000	1.000	*Veillonella parvula* (T);	X84005
(DNA)				DSM 2008;	
		1.000	1.000	*Veillonella* sp.	AF287782
				oral clone AA050	
14	371	1.000	1.000	*Enterococcus faecalis;*	AY692453
(DNA & RNA)				SL5	
15	371	1.000	1.000	*Lactobacillus paracasei* subsp.	AY369076
(DNA & RNA)				*paracasei*; Akira1;	
		1.000	1.000	*Lactobacillus casei*; LC3	AY675252
16	371	1.000	1.000	*Lactobacillus reuteri* (T);	X76328
(DNA & RNA)				DSM 20016 T;	
		1.000	1.000	*Lactobacillus frumenti* (T);	AJ250074
				TMW 1.666	
17	366	1.000	1.000	*Fusobacterium nucleatum*;	DQ440557
(DNA)				Ulm 8;	

* As deduced by comparison with fingerprints run on the same gel.

A comparison of DNA and RNA fingerprints showed commonalities but also some differences. Bands indicating the presence of members of the *Bacteroides* group (bands 1-4) were observed on all 8 fingerprints shown in figure 3.11, indicating those microorganisms to be present and active. Similarly, a prominent band of *Enterococcus faecalis* (14) is observed on both DNA and RNA fingerprints from the proximal liver ends of both samples and also bands originating from *Lactobacillus gasseri* (9) can be observed on DNA and RNA fingerprints of the distal liver ends of both stents. Bands for *Lactobacillus* sp. (15 and 16) are also present on all fingerprints, although on the RNA fingerprints these bands seem to be more pronounced at the distal liver end. In contrast, faint bands originating from *Escherichia coli* (6), *Bifidobacterium* sp. (7 and 8), *Veillonella* sp. (13) or *Fusobacterium* sp. (17) are only visible on DNA fingerprints but absent from on RNA fingerprints indicating those organisms to be present but not active. Bands showing a sequence closely related to *Streptococcus anginosus* (10 and 11) are present on all DNA fingerprints, but are only detectable on the RNA fingerprint of the distal liver end of stent B indicating those organisms to be dead or metabolically resting and to not contain enough ribosomal RNA to be detectable on the RNA fingerprints. Microorganisms closely related to *Prevotella nanceiensis* (5) are only present on 1 RNA fingerprint, namely at the proximal liver end of stent B, pointing to some activity of these bacteria in the biofilm, although the abundance of this group may be too low to be detectable on the DNA fingerprints.

3.4 Analysis of 16S rDNA clone libraries of biliary stents

For a more detailed characterisation of microbial community composition in biliary stent biofilms and to validate the use of SSCP fingerprinting with such samples, two biliary stents, one of them originating from BS (stent I) and the other from SH (stent II) were chosen for random sequencing of generated 16S rDNA clone libraries. Stent I was 11 cm in length and made from polyethylene with a diameter of 8.5 French (ca. 2.8 mm) and removed due to complete obstruction. Stent II was 12 cm in length and made from polytetrafluorethylene with a diameter of 10 French (ca. 3.3 mm) and removed for precautionary reasons. The residence time for both stents was approximately 3 months. Both patients suffered from tumours leading to stricture of the bile ducts. No prophylactic antibiotics were given to either patient.

3.4.1 Sequence analysis and phylogenetic classification

Of each library, 94 clones were picked randomly and submitted to 16S rDNA sequencing. After rejection of bad quality or low signal reads, 82 sequences of stent I (41 sequences generated from biofilm proximal to the liver, abbreviated as L and 41 sequences generated from biofilm distal to the liver, abbreviated as D) and 88 sequences of stent II (46L/42D) remained for analysis. Sequences were first checked for chimera or other sequence anomalies using Mallard (Ashelford *et al.*, 2006) and Bellerophon (Huber *et al.*, 2004), leaving 60 clones (32L/28D) of library I (stent I from BS) and 75 clones (39L/36D) of library II (stent II from SH). Thus, the overall occurrence of chimeric sequences in libraries I and II was 27% and 15%, respectively. The remaining sequences were aligned and the generated distance matrix was grouped into operational taxonomic units (OTUs) using the program DOTUR at a cut-off level of 1.5% difference. It is known that bacterial isolates that show less than 97% 16S rDNA sequence identity usually show less than 70% similarity in DNA-DNA hybridisation experiments, thus belonging to different species (Stackebrandt & Goebel, 1994). However, bacterial isolates sharing more than 97% of 16S rDNA sequence identity not necessarily share high similarity over the whole genome which may be significantly lower than the 70% level of DNA-DNA hybridisation (Fox *et al.*, 1992), currently accepted to classify different isolates as belonging to the same species (Gevers *et al.*, 2005). In current publications different cut-off levels between 1 and 3% difference in the 16S rDNA sequence are used to group sequences from random clone

libraries in OTUs (Bik *et al.*, 2006; Hayashi *et al.*, 2005; Wang *et al.*, 2005; Wang *et al.*, 2003) complicating the comparison of diversity between different communities. For bacterial communities from biliary stents examined in this work a cut-off level of 1.5% was chosen, in order to separate phylogenetic close species e.g. within the family of Enterobacteriaceae but still achieve a clustering of phylogenetic broader groups.

3.4.1.1 Bacterial community structure of library I

Figure 3.12 presents a phylogenetic tree of all sequenced clones of library I, including type and reference strains as well as anaerobic and aerobic isolates (see chapter 3.4.3) from stent I. Data analysis with DOTUR resulted in 12 OTUs when choosing a cut-off level of 1.5% difference. Seven of these belonged to the phylum of Firmicutes and the remaining 5 to the phylum of Proteobacteria with the majority of cloned sequences affiliated with the Firmicutes (53 out of 60). A large set of 20 clones was affiliated with the family of Streptococcaceae and could be clustered in three different OTUs containing 15, 4 and 1 cloned sequences, respectively. Clones affiliated with the family of Enterococcaceae were also highly abundant (13 clones) and were closely related to either *Enterococcus avium* or *Enterococcus faecalis*. Other abundant sequence types affiliated with the Firmicutes were related to *Finegoldia* sp. (12 sequences) and *Veillonella* sp. (8 sequences), respectively. The phylum of Proteobacteria is represented only by members of the class of gamma Proteobacteria. However, all OTUs detected (most closely related to *Klebsiella oxytoca, Citrobacter freundii, Citrobacter* sp., *Enterobacter* sp. and *Haemophilus* sp.) were present only in low abundance and are represented by only 1-2 sequences.

Interestingly, the OTUs represented by *Enterococcus* sp., *Veillonella* sp. and *Finegoldia* sp. seem to be dominated by sequences originating from the proximal liver end, whereas the OTUs represented by *Streptococcus* sp. seem to be dominated by sequences originating from the distal liver end of the stents.

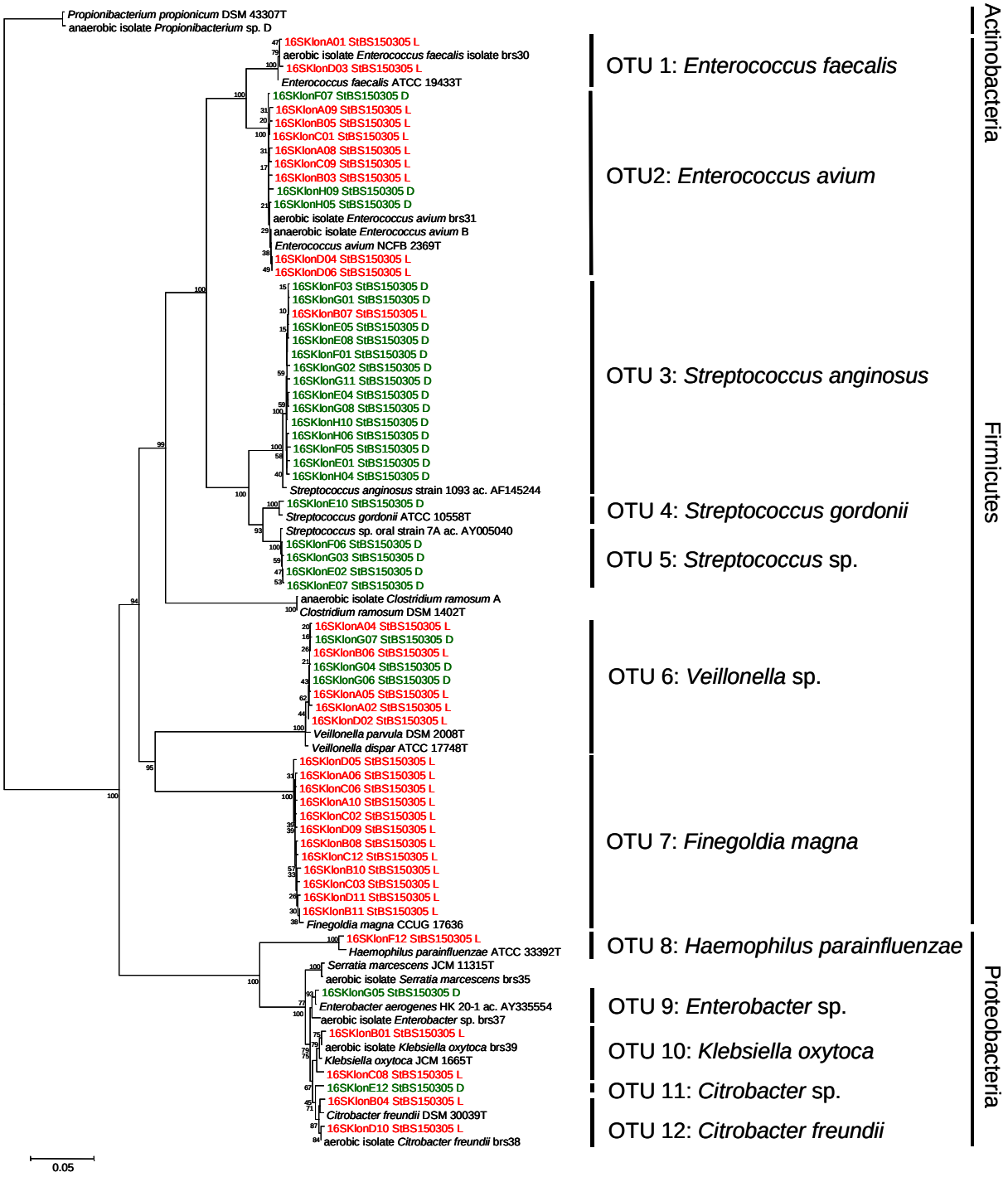

Figure 3.12: Phylogenetic tree showing the affiliation of 16S rDNA sequences cloned from stent I. Sequences originating from the proximal liver end are shown in red, sequences from the distal liver end in green and isolates and type and reference strains are in black. The tree was constructed by neighbour-joining analysis using a Jukes-Cantor correction. Bootstrap values (percentages of 100 replications) are shown at branch points. The scale bar represents the genetic distance (5 substitutions per 100 nucleotides). The right side of the figure shows the OTUs grouped by analysis with DOTUR using a cut-off level of 1.5% difference and the phyla the sequences are affiliated with.

3.4.1.2 Bacterial community structure of library II

Figure 3.13 presents the phylogenetic tree of sequences obtained from library II, also including type and reference strains. Clustering with DOTUR resulted in 9 OTUs when choosing a cut-off level of more than 1.5% difference. Like in library I, OTUs belonging to the phyla of Proteobacteria and Firmicutes were the most abundant (4 OTUs each), however, in library II also 1 sequence affiliated with the phylum of Bacteroidetes was observed. Also in contrast to library I, where the vast majority of sequences was affiliated with the Firmicutes, sequences affiliated with the Proteobacteria (48) were more abundant than those affiliated with the Firmicutes (26) in library II. Sequences belonging to the OTU represented by *Shigella* sp./*Escherichia coli* (34) were the most abundant in the library followed by 20 sequences belonging to the OTU represented by *Enterococcus faecalis*. Another abundant sequence type affiliated with the Firmicutes was related to *Veillonella* sp. (4 sequences), whereas among the Proteobacteria sequences related to *Klebsiella pneumoniae* (8 sequences) were also abundant. Other OTUs affiliated with the phylum of Proteobacteria are *Shigella boydii* (4 sequences) and *Klebsiella oxytoca* (2 sequences). Other OTUs affiliated with the phylum of Firmicutes are *Enterococcus casseliflavus* and *Anaeroglobus geminatus* (each 1 sequence). The Bacteroidetes phylum is represented by *Prevotella tannerae* (1 sequence).

Interestingly, the OTUs belonging to the Proteobacteria seem to be dominated by sequences originating from the distal liver end and the OTUs belonging to the Firmicutes seem to be dominated by sequences originating from the proximal liver end of the stent.

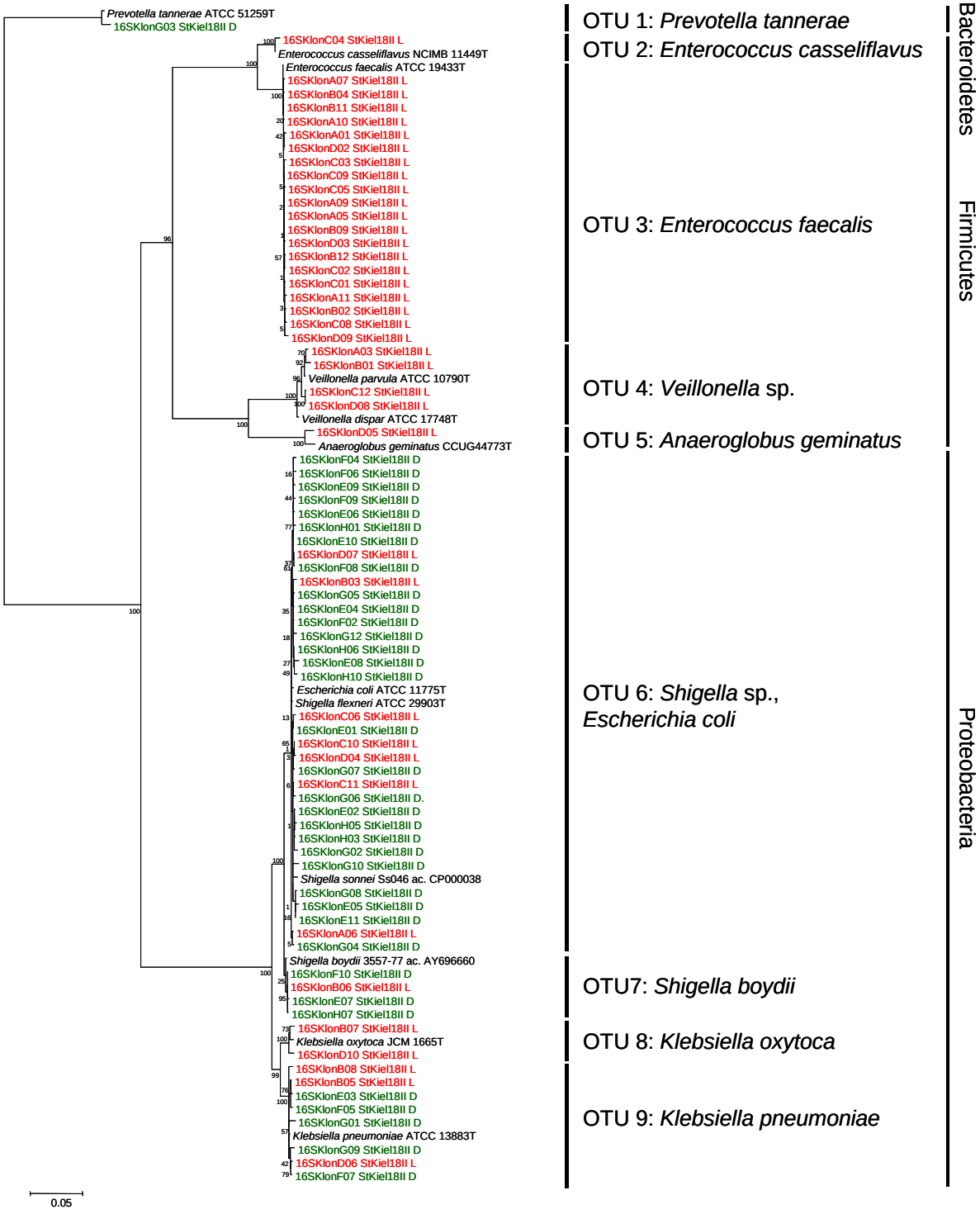

Figure 3.13: Phylogenetic tree showing the affiliation of 16S rDNA sequences cloned from stent II. Sequences originating from the proximal liver end are shown in red, sequences from the distal liver end in green and isolates and type and reference strains are in black. The tree was constructed by neighbour-joining analysis using a Jukes-Cantor correction. Bootstrap values (percentages of 100 replications) are shown at branch points. The scale bar represents the genetic distance (5 substitutions per 100 nucleotides). The right side of the figure shows the OTUs grouped by analysis with DOTUR using a cut-off level of 1.5% difference and the phyla the sequences are affiliated with.

3.4.1.3 OTUs observed in sequences from both libraries

In order to compare the phylogenetic composition of both biliary stent clone libraries, OTUs at a cut-off value of 1.5% difference were calculated with DOTUR for the combined set of sequences. As shown in figure 3.14, only 3 OTUs are shared by the two clone libraries, namely sequences related to *Enterococcus faecalis*, *Veillonella* sp. and *Klebsiella oxytoca*. Remarkably, the group of sequences most closely related to *Klebsiella oxytoca* split up in two OTUs. Since OTUs were calculated using the furthest neighbour algorithm, this indicates that the outgrouping sequence of clone library I differed more than 1,5% in its 16S rDNA sequence from at least one sequence of the respective OTU in library II, pointing to a high heterogeneity within this group. The observation that the two communities are not considerably overlapping is in accordance with former observations of major inter-subject differences when comparing the microbial community structure of habitats such as the mucosa of the colon or gut contents of different sections of the gastrointestinal tract in human individuals (e.g. (Hayashi *et al.*, 2005; Zoetendal *et al.*, 2002)). Furthermore, results of PCR-SSCP fingerprinting already had indicated a high variability between microbial communities observed in stents of different patients.

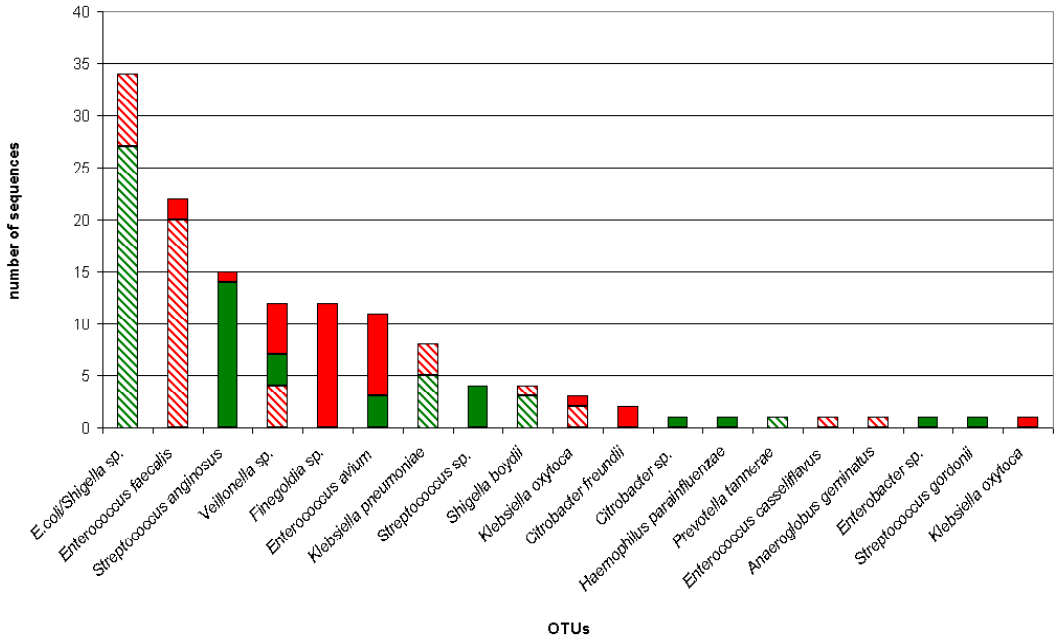

Figure 3.14: OTUs calculated with DOTUR using a cut-off value of 1.5% from the combined sequences of both 16S rDNA clone libraries. Red: stent I, proximal to the liver; Green: stent I, distal to the liver; Dashed red: stent II, proximal to the liver; Dashed green: stent II, distal to the liver.

3.4.1.4 Diversity measures

Diversity measures and richness estimators are useful for the comparison of the relative complexity of communities and to estimate in how far the obtained sequence diversity reflects the actual structure of communities. Rarefaction curves which plot the number of OTUs observed against the number of clones sequenced for both stents for which the community composition has been analysed by sequencing of 16S rDNA clone libraries are shown in figure 3.15. Evidently, the microbial community established in stent I is more diverse than that having established in stent II. Moreover, both the communities of the proximal and distal liver ends of stent I are more diverse than the respective communities of stent II. The slope of the rarefaction curves indicates the rate at which new OTUs are discovered in the respective libraries. In the case of the total library of stent I (green squares) no clear plateau in OTU detection rate is observed. This is due, as evident from figure 3.15, to the diversity of the community at the distal liver end of stent I where some phylogenetic groups remained undetected, while most of the phylogenetic diversity of the community at the proximal liver end has been detected. Respective analysis of the characterised stent II community (red squares) indicates that the obtained sequences cover the majority of the diversity in this community. The rarefaction curves for the proximal and distal liver end of sample II indicate that the community at the proximal liver end accounts for more diversity than the community at the distal liver end. Good's coverage (Good, 1953) for stent I was 93.3%, with 96.9% for the proximal and 85.7% for the distal liver end, thus supporting the observation obtained from rarefaction analysis. For stent II the coverage of Good was 96%, with 94.9% for the proximal and 97.2% for the distal liver end. Overall, these analyses indicated that the obtained sequence information gives realistic overviews on the respective community compositions.

The Chao-1 richness estimator (Chao, 1987) gives an estimation of the number of OTUs present in the actual microbial community. For stent I the actual microbial community is estimated to consist of 13.5 (12.2; 24.5) OTUs, whereas for stent II a number of 10.5 (9.1; 24.1) OTUs was estimated (95% confidence intervals are given in brackets). Although, a slightly different richness estimation was obtained for both libraries, the upper bounds of the confidence intervals are almost identical, suggesting a negligible difference in actual diversity.

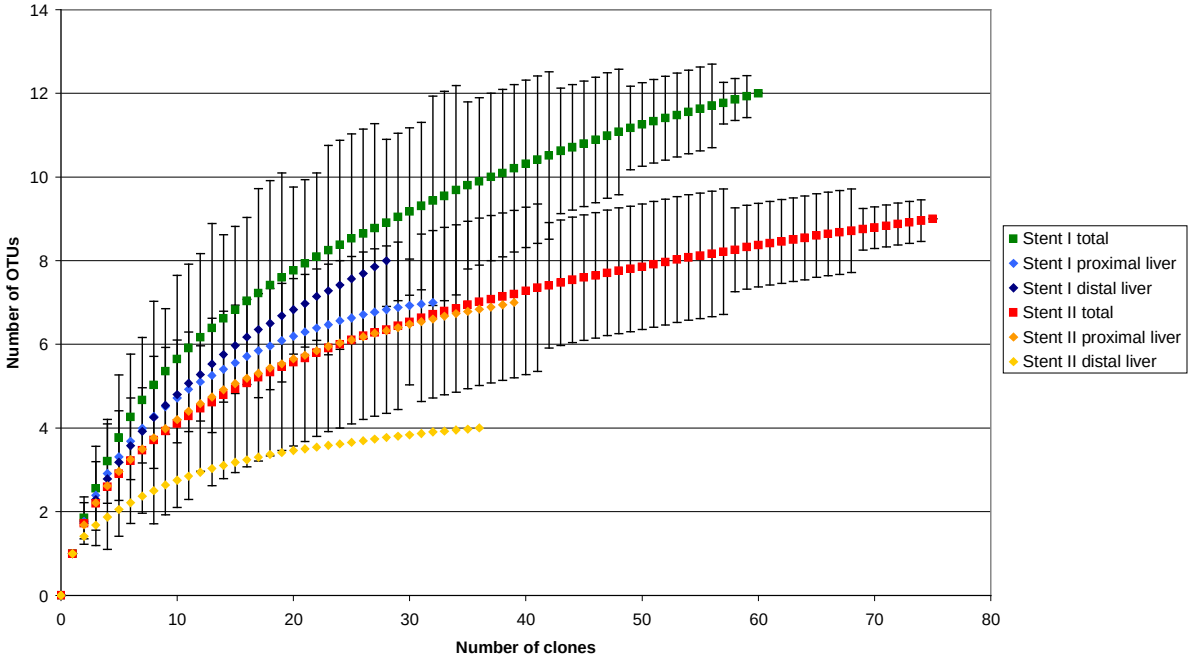

Figure 3.15: Rarefaction curves generated for 16S rDNA sequences of the clone libraries of stent I and II. Rarefaction analysis was performed on the total sequences of each stent, but also selectively for libraries generated from either the distal or proximal liver ends. For clarity, error bars indicating 95% confidence intervals are only displayed for rarefaction curves of total sequences of each stent. The slope of each curve indicates the rate at which new OTUs were discovered. OTUs are grouped at a level of sequence similarity ≥ 98.5%.

The diversity index of Shannon and the reciprocal of the Simpson index were calculated after grouping of the sequences into OTUs at cut-off levels of 1.5% and 2% sequence difference, with the last cut-off level being used to allow a comparison of this work with the study of Wang et al. describing the community of the human jejunum using cloning and sequencing of 16S rRNA genes (Wang *et al.*, 2005). The jejunum is the habitat that is the closest related to the human duodenum, which itself can be

regarded as the inoculum for biliary stent communities and of which no molecular study on bacterial microbiota is published to date. The Shannon index (H') is a general diversity index that takes into account the number of species and the evenness, whereas the Simpson index D is a dominance measure which represents the probability that two randomly selected sequences belong to the same OTU. The use of the reciprocal ensures that the value of the index increases with increasing diversity (see also materials and methods 1.8). Table 3.10 shows the diversity measures calculated, as well as the data of the jejunum library (Wang *et al.*, 2005). For stent I, H' and 1/D values of the partial libraries, proximal and distal to the liver, indicate a similar diversity, whereas the respective values of the partial stent II libraries differ significantly and indicate a substantially higher diversity at the proximal liver end. The diversity indices for the total library are similar to the ones for the proximal liver end, suggesting that this part of the stent contributes primarily to the diversity of the total stent community. Comparing the diversity measures for the libraries of stent I and II, a greater diversity is indicated for stent I (which was also observed in the rarefaction curve analysis). The diversity indices calculated for the combined sequences of both stents are considerably higher, indicating, as described above, only small overlaps in the respective communities. The number of OTUs calculated decreases with increasing cut-off levels applied. For library I the OTUs 9 & 10 and 11 & 12 and for library II the OTUs 6 & 7 (see figures 3.12 and 3.13) merge at a cut-off level of 2%, respectively.

Table 3.10: Diversity measures for OTUs calculated with ≥ 98.5% and ≥ 98.0% sequence similarity.

Sequence similarity		98.5%	98.0%	98.5%	98.0%	98.5%	98.0%
Community	Number of sequences	Number of OTUs		H' [1]		1/D [2]	
Stent I total	60	12	10	2.042	1.978	6.730	6.629
Stent I proximal liver	32	7	7	1.633	1.633	4.636	4.636
Stent I distal liver	28	8	7	1.579	1.530	3.670	3.635
Stent II total	75	9	8	1.532	1.361	3.504	2.990
Stent II proximal liver	39	7	7	1.439	1.439	3.250	3.250
Stent II distal liver	36	4	3	0.797	0.526	1.731	1.416
Stent I and Stent II	135	19	15	2.335	2.193	8.019	7.111
Jejunum (Wang *et al.*, 2005)	88		22		1.897		2.945

[1] Shannon index.
[2] Reciprocal of Simpson index.

In the 88 sequences generated by Wang *et al.* from biopsies of the human jejunum 22 OTUs were detected at a sequence similarity of ≥ 98.0%, with a Chao-1 estimation of 40 (26; 93) OTUs present (95% confidence intervals are given in brackets). The Chao-1 richness estimation gives 10.5 (10.0; 18.3) and 9.5 (8.1; 23.1) OTUs for library I and II at a cut-off level of 2% sequence difference, respectively. The diversity of the duodenum, which is the source for microorganisms invading biliary stents, is assumed to be lower compared to the jejunum due to the proximal-distal gradient in the human gut, showing an increase in OTU number from small to large intestine (Wang *et al.*, 2003). Thus, since the species richness observed in the jejunum compared to the species richness observed in this study is higher, a similar diversity of biliary stent biofilm communities and the duodenum may be assumed. H' and the 1/D were 1.897 and 2.945, respectively for the jejunum library. H' being similar to the observed indices of this study suggests a higher evenness for the biliary stent libraries, since observed species richness is lower. Accordingly, 1/D of the biliary stent libraries was found to be higher, suggesting a higher probability that two randomly selected clones will be of the same OTU, and therefore more OTUs, which are scarcely abundant in the jejunum library. However, as observed during SSCP fingerprinting, the microbial communities observed in stent biofilms of single patients represent only a fraction of the diversity observed in all stents. Thus, the diversity of the microbiota as observed in all stents is assumed to be more similar to the jejunum microbiota, than the diversity of single biofilm communities from biliary stents.

3.4.2 Comparison of biliary stent microbial community structure as revealed by random sequencing of 16S rDNA clone libraries and SSCP fingerprinting

To analyse in how far SSCP fingerprinting reflects the community structure revealed by 16S rDNA clone libraries, results obtained by both methods were compared. Figure 3.16 shows the SSCP fingerprints of stent I used for the construction of 16S rDNA clone library I. Bands for which a sequence could be retrieved are labelled. The according phylogenetic classification is summarised in table 3.11.

Analysis of the clone library of stent I had indicated the community at the distal liver end to be dominated by *Streptococcus anginosus* and other *Streptococcus* sp. sequences, *Veillonella* sp. and *Enterococcus avium* to be abundant. In fact, the predominant band observed after SSCP fingerprinting of the distal liver end (band 4) revealed a sequence identical to that of the Com fragment of the 16S rDNA clones of the OTU represented by *Streptococcus anginosus.* The Com fragments of the other two Streptococci observed in much lower abundance in the clone library differ in 10 (OTU 4, *Streptococcus gordonii*) and 17 (OTU 5: *Streptococcus* sp.) base pairs, respectively, from the Com fragment of *Streptococcus anginosus* and were not observed on the SSCP fingerprint. Also bands closely related to *Veillonella* sp. (band 5) and *Enterococcus avium* (band 6) were observed on the SSCP fingerprint.

The clone library obtained from the biofilm of the proximal liver end was dominated by sequences closely related to *Enterococcus avium* and *Finegoldia magna*. In accordance, the SSCP fingerprint generated also shows prominent bands indicating *Enterococcus avium* (band 7) and *Finegoldia magna* (band 8) to be highly abundant in that biofilm. Moreover, the band represented by *Finegoldia magna* appeared only on the SSCP fingerprint of the proximal liver end, identical to results of the 16S rDNA clone library where *Finegoldia magna* sequences were only observed in the respective sublibrary. A faint band observed was closely related to *Citrobacter freundii* (band 3), this is in accordance with a low abundant *Citrobacter* sp. OTU in the clone library. In summary, SSCP fingerprinting gives a reliable overview on the predominant species in the biofilm of stent I.

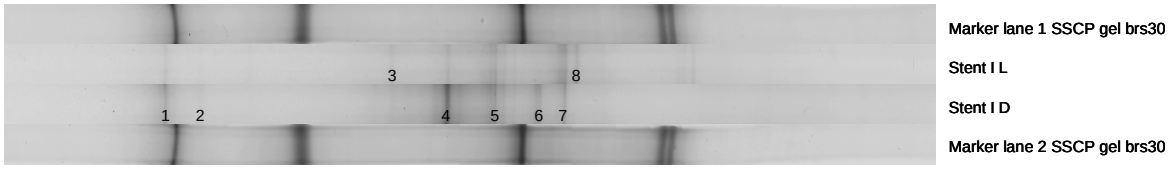

Figure 3.16: SSCP fingerprints (50 ng ssDNA/lane) of stent I for which a 16S rDNA clone library was generated. Abbreviations: L: side proximal to the liver, D: side distal to the liver. The marker lane consists of five bands corresponding to the Com fragment of the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

Table 3.11: Sequences reamplified from SSCP bands of stent I and their phylogenetic closest neighbour according to Sequence match of RDP II.

Sequence identity	Sequence length [1]	Similarity score [2]	Seqmatch score (S_ab) [3]	Phylogenetic closest neighbour	NCBI accession number
I - 1	370	1.000	1.000	*Klebsiella oxytoca,* ATCC 13182T	Y17655
I - 2	366	1.000	1.000	*Clostridium ramosum,* DSM 1402T	X73440
I - 3	370	1.000	1.000	*Citrobacter freundii*, DSM 30039T	AJ233408
I –4	370	0.995	0.961	*Streptococcus anginosus,* ATCC 33397	AF104678
I – 5	370	1.000	1.000	*Veillonella sp.* ADV	AY571668
I – 6	368	0.992	0.961	*Haemophilus parainfluenzae,* CCUG 12836	AY362908
I – 7	371	1.000	1.000	*Enterococcus avium,* LMG 10744	AJ301825
I – 8	366	1.000	1.000	*Finegoldia magna,* ATCC 29328	AB109770

[1] Excluding primer sequences.
[2] Percent identity over all pairwise comparable positions.
[3] Number of (unique) oligomers shared between the query and the subject sequence divided by the lowest number of unique oligos in either of the two sequences.

Figure 3.17 presents the SSCP fingerprints of the distal and proximal liver end of stent II. Bands for which a sequence could be reamplified are labelled and their phylogenetic classification is summarised in table 3.12. The SSCP fingerprint of the distal liver end

of stent II showed two predominant bands, the sequences of which are closely related to *Shigella* sp. and *Escherichia coli* (band 4 and 5). This is in good accordance with the analysis by random sequencing of the clone library, where the majority of sequences originating from the distal liver end were grouped in OTU 6 or 7, corresponding to *Shigella/Escherichia* species. The fingerprint of the proximal liver end also shows bands related to *Shigella* sp. and *Escherichia coli,* with the respective sequences also being observed in the corresponding clone library. Minor bands indicating the presence of microorganisms closely related to *Klebsiella pneumoniae* (band 1) and *Klebsiella oxytoca* (band 3) are observed on the fingerprints. In accordance 16S rDNA clones showing a sequence similarity of 100% and 99%, respectively, were identified. Furthermore, bands indicating the presence of *Enterococcus casseliflavus*, *Veillonella* sp. and *Enterococcus faecalis* (bands 6, 7 and 8) were observed exclusively on the fingerprint of the proximal liver end. Accordingly, respective sequences were observed exclusively in the clone library of the proximal liver end. Thus, as stated above for stent I, SSCP fingerprinting gives a reliable overview on predominant species of the respective biofilm communities. In conclusion, by detailed analysis of two stent samples through random sequencing of 16S rDNA libraries, SSCP fingerprinting was validated as reliable method for analysis of microbial communities of biliary stent biofilms.

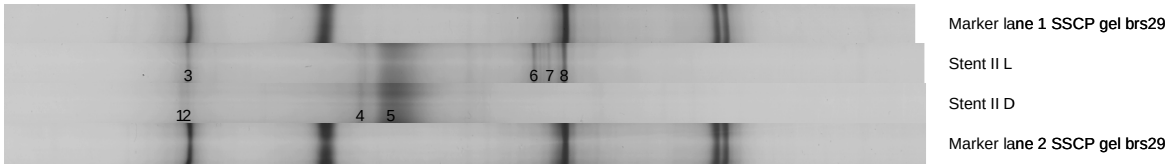

Figure 3.17: SSCP fingerprints (50 ng ssDNA/lane) of stent II for which a 16S rDNA clone library was generated. Abbreviations: L: side proximal to the liver, D: side distal to the liver. The marker lane consists of five bands corresponding to the Com fragment of the following isolates: *Klebsiella oxytoca, Staphylococcus epidermidis, Enterococcus faecalis* and *Agrobacterium radiobacter* (double band).

Table 3.12: Sequences reamplified from SSCP bands of stent II and their phylogenetic closest neighbour according to Sequence match of RDP II.

Sequence identity	Sequence length [1]	Similarity score [2]	Seqmatch score (S_ab) [3]	Phylogenetic closest neighbour	NCBI accession number
II – 1	370	1.000	1.000	*Klebsiella pneumoniae*, DSM 30104T	X87276
II – 2	370	1.000	1.000	*Enterobacter aerogenes*	AF395913
		1.000	1.000	*Pantoea agglomerans,* A8	AF130960
II – 3	370	1.000	1.000	*Klebsiella oxytoca,* ATCC 13182T	Y17655
II – 4	370	1.000	1.000	*Shigella boydii*	X96965
		1.000	1.000	*Escherichia coli*	Z83204
		1.000	1.000	*Shigella flexneri*	X96963
		1.000	1.000	*Shigella sonnei*	X96964
II – 5	370	1.000	1.000	*Shigella boydii*	X96965
		1.000	1.000	*Escherichia coli*	Z83204
		1.000	1.000	*Shigella flexneri*	X96963
		1.000	1.000	*Shigella sonnei*	X96964
II – 6	371	1.000	1.000	*Enterococcus casseliflavus,* NCBI 11449	Y18161
		1.000	1.000	*Enterococcus gallinarum,* LMG 13129	AJ301833
II – 7	304 (partial sequence)	0.990	0.933	*Veillonella dispar,* DSM 20735	X84006
		0.990	0.933	*Veillonella parvula,* ATCC 17745	*AY995769*
II – 8	371	1.000	1.000	*Enterococcus faecalis,* SFL	AY850358

[1] Excluding primer sequences.
[2] Percent identity over all pairwise comparable positions.
[3] Number of (unique) oligomers shared between the query and the subject sequence divided by the lowest number of unique oligos in either of the two sequences.

3.4.3 Isolating and sequencing members from biliary stent communities

Aerobic and anaerobic enrichment and isolation was carried out with stent I from BS. Isolation from stent II from SH was not possible since the sample was frozen for transport. A comparison with the two culture-independent methods appears to be interesting since former studies on biliary stent biofilm composition were based on culturing methods (Di Rosa *et al.*, 1999; Dowidar *et al.*, 1991; Leung *et al.*, 2000b; Molinari *et al.*, 1996; Speer *et al.*, 1988b; Zhang *et al.*, 2003), even though it is well established that enrichment and culturing from microbial communities implies a strong bias. Tables 3.13 and 3.14 show isolates obtained by enrichment under aerobic or anaerobic conditions and their identification as revealed by 16S rDNA sequencing.

Table 3.13: Aerobic isolates of stent I and their phylogenetic closest neighbour according to Sequence match of RDP II.

Isolate	Sequence length [1]	Similarity score [2]	Seqmatch score (S_ab) [3]	Phylogenetic closest neighbour	NCBI accession number
brs 30	1483	1.000	1.000	*Enterococcus faecalis*; ATCC 19433	DQ411814
brs 31	1481	1.000	1.000	*Enterococcus avium*; ATCC 14025	DQ411811
brs 35	1464	0.999	0.999	*Serratia marcescens* subsp. *marcescens*; AU1209	AY043387
brs 37	1464	0.999	0.995	*Enterobacter* sp. B901-2	AB114268
brs 38	1465	0.999	0.976	*Citrobacter freundii*; HQ010516B-1	DQ010114
brs 39	1462	1.000	0.997	*Klebsiella oxytoca*; strain 5	DQ294284

[1] Excluding primer sequences.
[2] Percent identity over all pairwise comparable positions.
[3] Number of (unique) oligomers shared between the query and the subject sequence divided by the lowest number of unique oligos in either of the two sequences.

Table 3.14: Anaerobic isolates of stent I and their phylogenetic closest neighbour according to Sequence match of RDP II.

Isolate	Sequence length [1]	Similarity score [2]	Seqmatch score (S_ab) [3]	Phylogenetic closest neighbour	NCBI accession number
brs A	1449	1.000	0.990	*Clostridium ramosum*; M91	AY699288
brs B	1481	1.000	1.000	*Enterococcus avium*; ATCC 14025	DQ411811
brs D	1444	0.999	0.984	*Propionibacterium* sp. H456	AB177643

[1] Excluding primer sequences.
[2] Percent identity over all pairwise comparable positions.
[3] Number of (unique) oligomers shared between the query and the subject sequence divided by the lowest number of unique oligos in either of the two sequences.

Tables 3.13 and 3.14 show that the relatively easy to culture members of the Enterobacteriaceae are covered quite well. Also several Enterococci could be isolated. The obtained 16S rDNA sequences of isolated bacteria are also incorporated in the phylogenetic tree of clone library I (figure 3.12) revealing that the three Enterococci isolates as well as the *Klebsiella oxytoca* and *Citrobacter freundii* isolate show more than 99% sequence identity with 16S rDNA clones. The sequence of the *Enterobacter* sp. isolate is 98.5% identical to its most related 16S rDNA clone. For the other isolates no significantly similar 16S rDNA clone sequences were observed. Streptococci and fastidious anaerobes like *Veillonella* sp. were not isolated here, although they were detectable using culture-independent methods. A sequence identical to the anaerobic isolate *Clostridium ramosum* was detected during SSCP fingerprinting, but not during random sequencing of 16S rDNA clone libraries. However, the weak band on the SSCP fingerprint indicates a low abundance of this microorganism in the community. Similarly, the isolation of microorganisms not predominant in the biofilm such as *Serratia marcescens* or *Propionibacterium* sp., as deduced by culture-independent methods, points to an enrichment of minor community members during cultivation.

3.5 Bile acid modifications in biliary stent biofilms?

As described in the introduction, several bacterial bile acid modifications are known from the large bowel. Through bacterial colonisation of the biliary tree, these metabolic transformations may also take place in the bile ducts, where the concentration of bile acids is high. The presence of the potential to carry out such transformations was tested in several approaches.

3.5.1 Bile salt hydrolase activity of biliary stent isolates

To elucidate whether bacterial bile salt hydrolase activity is present in biliary stent biofilms, a plate assay based on precipitation of deconjucated bile acids was performed with biliary stent isolates. The strain *Lactobacillus plantarum* LP80 (Christiaens *et al.*, 1992) (obtained by Prof. Verstraete) was used as a positive control and showed a differing morphology (intensively white and crystallised colonies) and clear precipitation on plates containing 0.1 and 0.5% of taurodeoxycholic acid (TDCA) compared to De Man, Rogosa and Sharpe (MRS) plates. As shown in table 3.15, 4 out of the 9 isolates tested exhibited bile salt hydrolase activity. All 4 isolates with bile salt hydrolase activity belonged to the genus *Enterococcus* and are characterised by a 16S rDNA sequence identical to SSCP phylotypes 43 (*Enterococcus faecalis*), 44 (*Enterococcus faecium*) and 52 (*Enterococcus avium*), respectively, in the region of the Com fragment. These 3 SSCP phylotypes include all SSCP phylotypes observed for Enterococci and were present in 36%, 34% and 17% of all fingerprints, respectively, and can thus be regarded as quite abundant members of biliary stent biofilm communities. The bsh positive enterococcal isolates showed a clear precipitation on 0.1 and 0.5% TDCA plates, being more pronounced on the higher concentrated plates. As an example two bsh positive strains are shown in figures 3.18 and 3.19. Both *Streptococcus* strains showed a growth inhibition on 0.5% TDCA plates.

Table 3.15: Growth and bile salt hydrolase activity of bacterial isolates on MRS and MRS supplemented with taurodeoxycholic acid plates.

Bacterial isolate	Growth	Precip.[1]	Growth	Precip.	Growth	Precip.
	MRS plates		MRS + 0.1% TDCA		MRS + 0.5% TDCA	
Lactobacillus plantarum LP80 (control)	+	-	+	+	+	+
Enterobacter sp. brs37	+	-	+	-	+	-
Enterococcus avium brs31	+	-	+	+	+	+
Enterococcus faecalis brs 23	+	-	+	-	+	-
Enterococcus faecalis brs30	+	-	+	+	+	+
Enterococcus faecalis brs51	+	-	+	+	+	+
Enterococcus faecium brs54	+	-	+	+	+	+
Streptococcus anginosus brs61	+	-	+	-	-	-
Streptococcus salivarius brs43	+	-	+	-	-	-
Pediococcus acidilactici brs52	+	-	+	-	+	-

[1] Precip. = Precipitation.

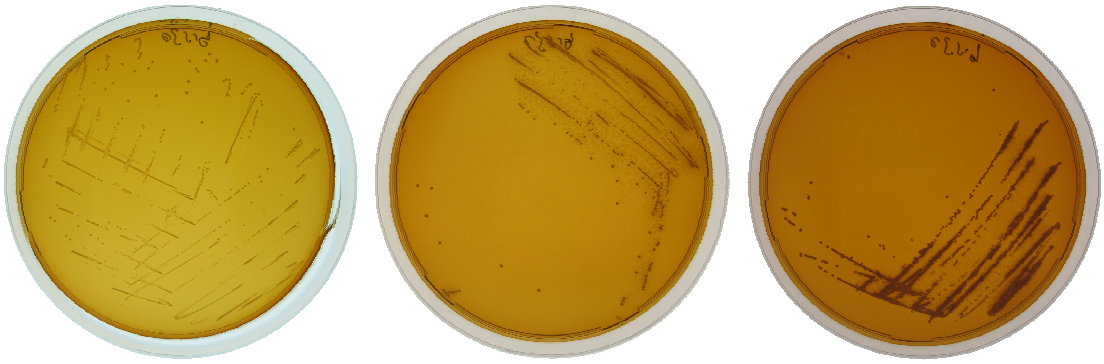

Figure 3.18: Growth of the biliary stent isolate *Enterococcus faecalis* brs30 on MRS, MRS + 0.1% TDCA and MRS + 0.5% TDCA plates (from left to right).

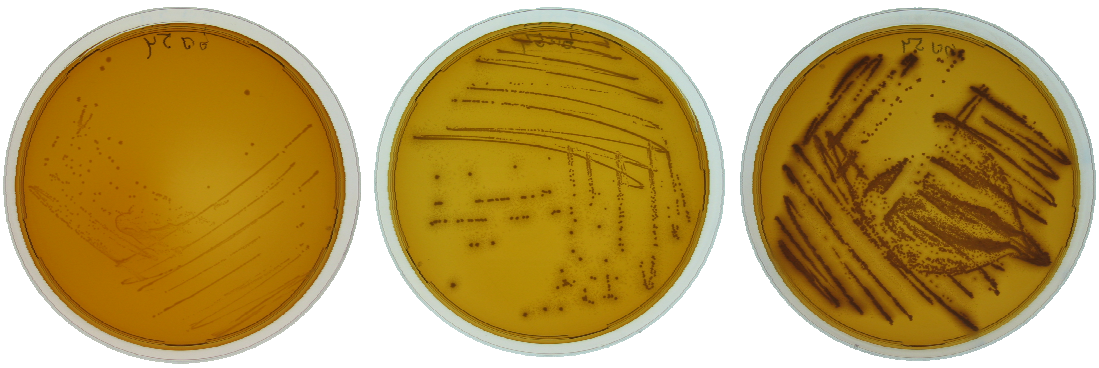

Figure 3.19: Growth of the biliary stent isolate *Enterococcus faecium* brs54 on MRS, MRS + 0.1% TDCA and MRS + 0.5% TDCA plates (from left to right).

3.5.2 Bai genes/7α-dehydroxylation

An additional bacterial transformation of bile acids is the 7α-dehydroxylation of primary to secondary bile acids. A PCR assay targeting the *baiCD* gene of two *Clostridium* sp., within the bile acid inducible operon encoding for products required for 7α-dehydroxylation of bile acids was developed recently (Wells *et al.*, 2003). This assay was performed with the total community DNA extracted both from proximal and distal liver ends of 3 stents from SH and 2 stents from BS. Furthermore, a *Clostridium perfringens* isolate of a biliary stent biofilm and the reference strain *Clostridium scindens* DSM 5676 were tested with the PCR assay. As shown in figure 3.20, a PCR product of approximately 1.3 kb in length was observed when DNA extracted from *Clostridium scindens* DSM 5676 was used as template. No such product was observed when stent community DNA had been used as template. Sequencing of some smaller PCR products which were observed to be formed in some cases revealed no similarity to known *bai* genes. Spiking of stent community DNA with DNA of *Clostridium scindens* DSM 5676 resulted in amplification of the expected fragment, thus inhibitory influences of stent community DNA can be excluded. In conclusion, *baiCD* genes amplifiable with primers designed for the amplification of such genes from Clostridia were absent in the 7 biliary stent communities analysed.

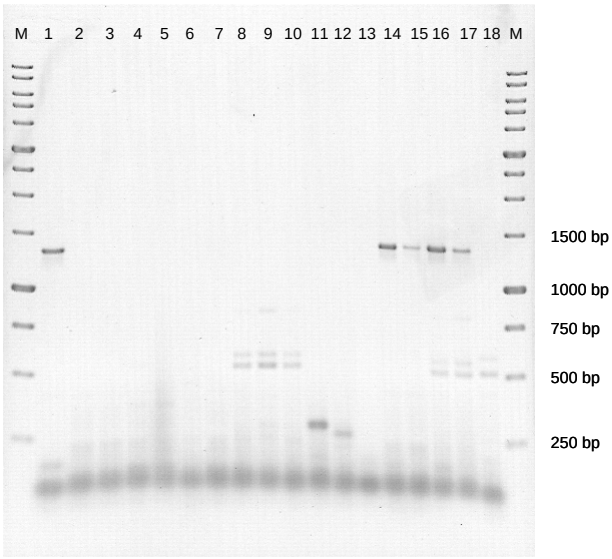

Figure 3.20: Assessment of the presence of the *baiCD* gene in biliary stent communities. PCR products obtained from DNA extracted from *Clostridium scindens* DSM 5676 (lane1, positive control, 20ng genomic DNA), 3 biliary stents of University Medical Center of Schleswig-Holstein in Kiel (lane 2-7, 200ng stent community DNA, proximal and distal liver ends), 2 biliary stents of Surgery Clinic of Braunschweig (lane 8-12, 200ng stent community DNA, stent I: proximal liver end, middle section of the stent, distal liver end; stent II: proximal and distal liver end) were separated on a 1% agarose gel. M: Marker lane (1kb Ladder, Applichem), lane 13: no template control, lanes 14+16: 200ng stent community DNA samples spiked with 20ng *Clostridium scindens* DSM 5676 DNA, lanes 15+17: 200ng stent community DNA samples spiked with 2ng *Clostridium scindens* DSM 5676 DNA, lane 18: biliary stent *Clostridium perfringens* isolate.

3.5.3 Taurine as electron acceptor

The hydrolysis of taurodeoxycholic acid, which is an abundant component of bile, generates taurine, which can be used as electron acceptor in an anaerobic respiration by *Bilophila wadsworthia* with ammonia, acetate and sulphide as products (Laue *et al.*, 1997). Interestingly, SSCP fingerprints of one patient from SZ (stents 0111-1020 I and II) revealed the presence of a band with a sequence identical to that of *Bilophila wadsworthia*, indicating microorganisms related to this bacterium to be abundant in this biofilm. As bile can be regarded as an ecological niche for taurine respiring organisms it was aimed to analyse if *B. wadsworthia* was present in lower abundance also in other bioflms. A specific PCR assay using non-degenerated primers to detect the taurine:pyruvate aminotransferase (*tpa*) gene of the degradative pathway for taurine has recently been developed and was proposed also as promising tool for rapid detection of *B. wadsworthia* in biological samples (Laue *et al.*, 2006). This assay was applied to biliary stent community DNA to elucidate the presence of this metabolic pathway in other stents. As shown in figure 3.21, a PCR product of the expected approximately 1 kb in length was observed when DNA extracted from the stent community characterised by the presence of *B. wadsworthia* was used as template. Such products were not observed in any of 7 other stents of SZ analysed in this respect. This indicates the absence of organisms with a taurine respiratory pathway similar to the one reported in *B. wadsworthia* in these biofilms.

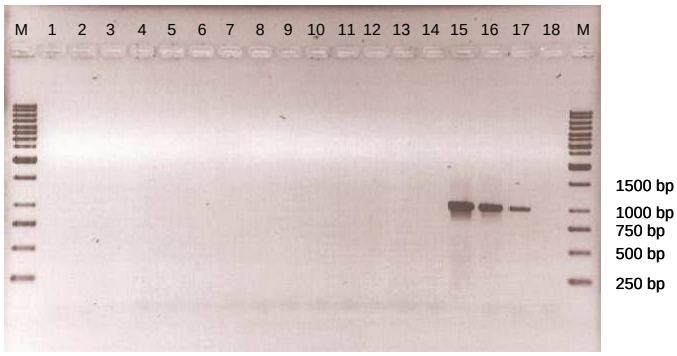

Figure 3.21: Assessment of the presence *tpa* genes in biliary stent communities. PCR products obtained from 10ng community DNA of 7 stents from Medical Clinic Salzgitter (lane 1-14) and stent 0111-1020 (positive control containing *Bilophila wadsworthia* 10, 1 and 0.1 ng stent community DNA, respectively; lane 15-17) are run on a 1% agarose gel. M: Marker lane (Gene Ruler 1kb Ladder, Fermentas), lane 18: no template control.

3.6 Transmission electron and light microscopy studies of two biliary stent biofilms

In order to gain insights into the structural organisation of biliary stent biofilms, 2 biliary stents from SZ implanted simultaneously, which had also been analysed by 16S rDNA and 16S rRNA SSCP fingerprinting (see section 3.3.7), were further analysed by sectioning and microscopy. As shown in figure 3.22 A, light microscopy revealed dietary fibers as well as bacterial microcolonies to be present in the biofilm. Dietary fibers may be introduced into the stents by duodenal reflux into the heptobiliary system and later contribute to obstruction of the stents. On the electron micrograph of stent I (fig. 3.22 B) yeast cells are visible (shown by the arrow). Yeast cells were only observed in samples prepared from biofilm of the distal liver end of stent I. This corresponds well with the results of SSCP fingerprinting, where bands closely related to *Candida* sp. were only observed on the fingerprint of stent I distal to the liver. The electron micrograph of stent II (fig. 3.22 C) presents pleomorphic, probably gram positive bacteria, showing a thick layer of cell walls and/or extracellular polymeric substances (EPS, see arrow). On the electron micrograph shown in figure 3.22 D dietary fibers are observed which are crossed by a channel colonised by bacteria (arrows).

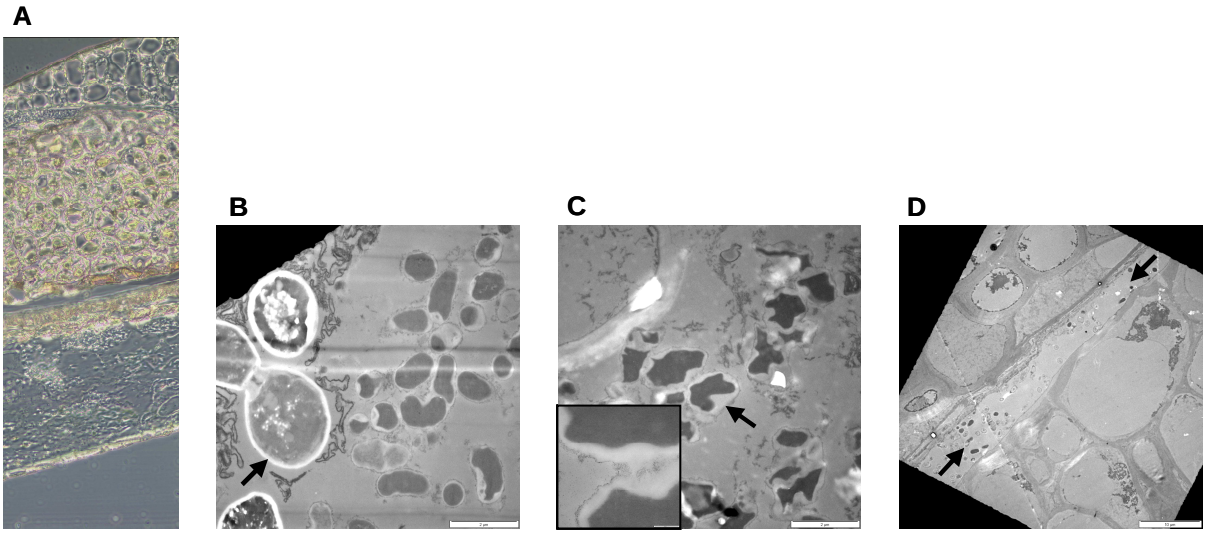

Figure 3.22: Light microscopy (A) and transmission electron micrographs (B-D) of the duodenal end of two stents of Salzgitter hospital.

In Figure 3.23 A-D electron micrographs of the proximal liver end of both stents are presented. The micrograph A shows an overview of the biofilm which grew on the surface of stent II. The biofilm observed in stent I (figure 3.23 B) consists predominantly of injured cells (arrows). The observation of mainly injured cells indicates the bacteria present to be in a resting state or dead and probably metabolic inactive. This is in good accordance with 16S rDNA and 16S rRNA fingerprinting, where several bands were observed exclusively on 16S rDNA fingerprints, and not on 16S rRNA fingerprints, indicating metabolically resting cells. Structures, which are in close contact with bacterial cells having an intensively stained acidic EPS layer (possibly mucine sheets, arrows) were observed in stent II (figure 3.23 C). These structures may help the microorganisms during attachment to the stent walls, thus enhancing biofilm formation and accelerating occlusion of the stents. A human lymphocyte as part of the host response to the biofilm was observed in stent I (figure 3.23 D).

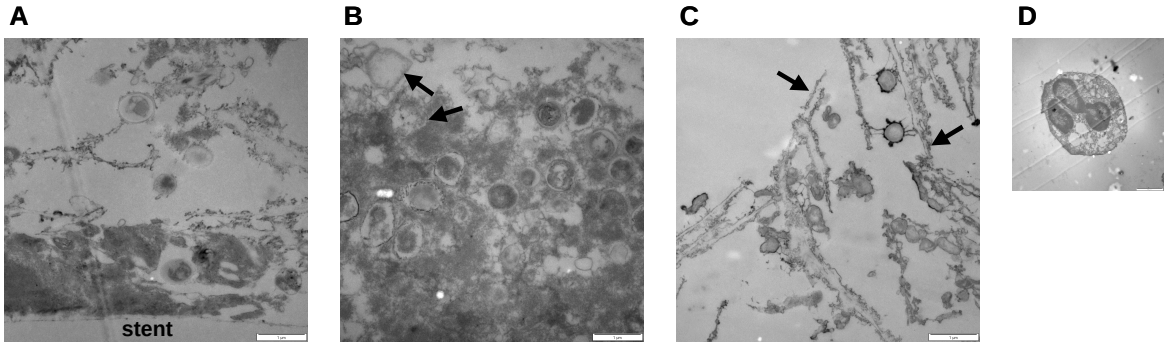

Figure 3.23: Transmission electron micrographs of the proximal end of two stents of Salzgitter hospital.

4 Discussion

4.1 Diversity and dynamics of microbial communities across stent biofilms

Overall, 62 bacterial SSCP phylotypes were identified across 133 fingerprints of microbial communities of stents from the Surgery Clinic of Braunschweig (herein abbreviated to BS) and the Medical Clinic of Salzgitter-Lebenstedt (herein abbreviated to SZ). These phylotypes cover 6 different bacterial phyla and represent a much broader diversity than those observed in previous studies based on culture-dependent methods (Di Rosa *et al.*, 1999; Dowidar *et al.*, 1991; Leung *et al.*, 2000b; Molinari *et al.*, 1996; Speer *et al.*, 1988b; Zhang *et al.*, 2003). In contrast to these culture-dependent studies, a higher abundance of anaerobic bacteria were observed (particularly, *Fusobacterium* sp., *Veillonella* sp. and *Bacteroides* sp.). This owes to the fact that this study did not have to isolate these microorganisms, for which isolation is not straightforward, in order to be able to characterise them. Also, a high abundance of Bifidobacteria and Lactobacilli was observed. However, the two most abundant phylotypes were *Veillonella* sp. (SSCP phylotype 41) and *Bifidobacterium* sp. (SSCP phylotype 2) occurring in more than 60% and 50% of all microbial communities, respectively. These species are typical members of the duodenal microbiota, although they are claimed to occur only in small numbers (Wilson, 2005). Other abundant phylotypes occurring in more than 30% of all analysed microbial communities were *Streptococcus anginosus* (SSCP phylotype 35), *Fusobacterium nucleatum* (B) (SSCP phylotype 54), *Enterococcus faecalis* (SSCP phylotype 43), *Enterococcus faecium/durans* (SSCP phylotype 44) and *Escherichia coli* (SSCP phylotype 23). Of these, the acid-tolerant *Streptococcus anginosus* is a major coloniser of the duodenum. Although the other above mentioned microorganisms are also colonisers of the small intestine, they have been found in higher numbers in the jejunum and the ileum than in the duodenum (Wilson, 2005). The ecological significance of the microorganisms predominant in biliary stent biofilms is discussed in chapter 4.3.

When the phylogenetic information obtained for single communities was summarised in a combined abundance of phylotypes, the biliary stent microbial community was

observed to be similar to the microbiota of the duodenum, where the prevalence of Streptococci, Lactobacilli, Enterococci and Enterobacteriaceae has been determined using culture-dependent methods (Bernhardt & Knoke, 1989; Skar *et al.*, 1989; Thadepalli *et al.*, 1979). Anaerobic bacteria detected have been largely comprised of Bacteroidetes, Fusobacteria and Clostridiae (Bernhardt & Knoke, 1989; Skar *et al.*, 1989). In some cases *Candida* sp. was observed (Bernhardt & Knoke, 1989; Skar *et al.*, 1989). A more recent culture-dependent study indicated a high prevalence of *Veillonella* sp. (Zilberstein *et al.*, 2007). However, since no culture-independent study of the duodenal microbiota is published to date, a comparison with available data from the closest habitat of microbes in humans, namely from the human jejunum was made (see chapter 3.3.4.2). A higher prevalence of Firmicutes and a lower prevalence of Proteobacteria and Actinobacteria were observed in the jejunum community compared to the combined abundance of phyla detected in all stents. This seems reasonable since the number of Proteobacteria decreases along the length of the intestine, whereby an increase in strict anaerobes is matched by the decrease in facultative anaerobes (Hayashi *et al.*, 2005; Marteau *et al.*, 2001). The increased number of Actinobacteria observed in biliary stent biofilms is in accordance with a higher abundance of this phylum in the upper digestive system, namely the oral cavity and the esophagus (Pei *et al.*, 2004; Wilson, 2005) and the supposed provenance of biliary stent biofilm members from the upper small intestine (Liu *et al.*, 1996; Sung, 1995).

4.1.1 Diversity and dynamics at the hospital level

Multivariate statistical analysis of the microbial communities across both BS and SZ revealed that any influences on microbial composition due to hospital effects were minor. That is, outpatient procedure versus hospitalisation or slightly different antibiotic use did not substantially influence the microbial community composition. The only apparent difference was a higher prevalence of Bifidobacteria in biofilms from SZ stents. Bifidobacteria are autochthonous bacteria in the gastrointestinal tract (Klijn *et al.*, 2005). Culture-independent studies on the small intestine found them in low abundance in that ecological niche (Hayashi *et al.*, 2005; Wang *et al.*, 2005), whereas they were more abundant in the colon and have been reported to comprise 3.3% in faeces (Vaughan *et al.*, 2002). Faecal composition changes with increasing age, where a decline in total number of bifidobacteria was matched with a decline in species

diversity in elderly subjects (Hebuterne, 2003; Woodmansey *et al.*, 2004). However, in biofilms from BS stents, for which patient data was available, no correlation between the occurrence of Bifidobacteria and age was observed.

Bifidobacteria are not naturally found in food but are purposely added because of their claimed health effects (Kullen & Bettler, 2005; Saarela *et al.*, 2000; Vaughan *et al.*, 2002). After ingestion of such preparations, bifidobacteria can be found alive in the gastrointestinal tract, however, those allochthonous bacteria do not persist for long periods of time (Kullen *et al.*, 1997). It has been reported that among the 10 taxa tested, cultures of *Bifidobacterium animalis* were the most capable to survive gastric transit (Masco *et al.*, 2007). While probiotics are live microbial food supplements, prebiotics are non-digestible short-chain carbohydrates that supposedly beneficially alter the composition or metabolism of the hosts gut microbiota (Macfarlane *et al.*, 2006) and it has emerged that some of them, largely oligosaccharides, have the potential to modify the microbiota in the human large bowel, both in the lumen and associated with the mucosal surface, resulting in an increase of Bifidobacteria and Lactobacilli (Langlands *et al.*, 2004; Macfarlane *et al.*, 2006). While an increase in the abundance of Bifidobacteria related to *B. animalis* in biliary stent biofilms following direct intake of probiotic bacteria seems to be plausible, regarding the short transit time of duodenal contents it is highly improbable that ingestion of prebiotics is able to influence the community composition of biliary stent biofilms. However, at least in the canteen kitchen of SZ neither probiotics nor prebiotics are used. On the other hand, at SZ many patients are treated in an outpatient procedure and the influence of nutrition is difficult to assess.

4.1.2 Diversity and dynamics at the patient level

Overall comparisons in microbial community structure of stents revealed that stent biofilms differed from individual to individual irrespective of hospital. That is, the community structures between several stents from the same patient (implanted either consecutively as is performed in BS or implanted simultaneously as is performed in SZ) were more similar to each other than to those of stents from other patients. However, patients from SZ who have had several simultaneous stents implanted have more similar microbial community structures across their stents than patients from BS who

have had several consecutive stents implanted and then removed. The distribution patterns of Bifidobacteria and *Veillonella* sp. accounted for such similarity between stent biofilms from the same patients who had simultaneous stents implanted, while the distribution patterns of *Fusobacterium nucleatum* (B), V*eillonella* sp. and *Streptococcus anginosus* accounted for such similarity between stent biofilms from the same patients who had consecutive stents implanted. The relationship between these species is further discussed in chapter 4.3.

The increased similarity between stents from the same patient seems reasonable because the stents are assumed to be inoculated by an ascending infection of the duodenal microbiota (Donelli *et al.*, 2007; Liu *et al.*, 1996; Sung, 1995), and the microbiota of various regions of the gastrointestinal tract are characterised by a strong host dependency. Thus, it is hypothesised that similar microbial biofilm communities will form across several stents. However, because the duodenal microbiota is subjected to major changes over time, due to rapid peristalsis and the short transit time of the lumenal contents (Wilson, 2005), it is hypothesised that stents implanted consecutively (instead of simultaneously) will show a larger variation in community composition. However, the biliary system which is sterile under normal conditions (Csendes *et al.*, 1975; Scott, 1971) is probably already colonised with microorganisms following the placement and removal of a preceding biliary stent (Matsuda *et al.*, 1991), thus the same microorganisms may colonise succeeding stents. That is, stents implanted consecutively will still show a similar diversity.

Strong host-dependency is in accordance with other studies on gastrointestinal microbial communities where pronounced inter-individual influences have been observed (Eckburg *et al.*, 2005; Hayashi *et al.*, 2005; Zoetendal *et al.*, 2002) and can be explained by differences in host physiology, host genetic background or environmental factors. For example, it has been reported that microbial communities of monozygotic twins have a higher similarity to each other than their marital partners, indicating a strong influence from the host genotype (Zoetendal *et al.*, 2001). Similarly, Lay *et al.* observed a large individual variability, which could not be explained by geographic origin, age or gender (Lay *et al.*, 2005). To exclude these host-related influences, a large within-subject sample size and multiple baseline samples are recommended for analysis of treatment effects on microbial communities of the intestine (Engelbrektson *et al.*, 2006). However, when dealing with patients having

biliary stents suffering from severe diseases such as pancreatic tumours this is impractical. Although there is a significant impact of host genotype on the bacterial composition in the gastrointestinal tract, environmental factors should not be neglected. A study on intestinal microbiota composition on 230 individuals at four European locations could show an association between geographic affiliation and differences in microbiota composition (Mueller *et al.*, 2006). Moreover, as microbial growth and metabolism in the intestine depend to a large extend on the supply of dietary carbohydrates that resist digestion in the upper tract, it is conceivable that dietary changes have an impact on microbial community composition, and various reports are available proving the stimulation of certain gut bacteria by dietary supplements (Duncan *et al.*, 2003; Gibson *et al.*, 1995; Kleessen *et al.*, 2001).

In summary, the high variance of microbial communities of different patients and the good overlap between total phylotypes observed and the assumed human duodenal microbiota, indicates an almost random colonisation of biliary stents depending on the prevailing duodenal microbiota of the patient at the time of stent placement. A comparison of culture-independent data of the duodenal microbiota with the observed combined abundances in biliary stent biofilms would be worthwhile, however currently such data is unavailable. Also, patient characteristics for which information was available, such as time of implantation or age of the patients did not correlate with the microbial community structures observed. Still, it would be interesting to compare microbial community composition to other parameters such as blood values of inflammatory markers. However, such data were unavailable at the time of study.

4.1.3 Diversity and dynamics at the stent level

Microbial community structure also differed along the length of stents and between biliary and pancreatic stents. When the combined abundances of SSCP phylotypes detected at the different sections of the stents were compared, the higher prevalence of Lactobacilli at the end distal to the liver for samples from both hospitals was the only apparent difference. This may be due to the more acidic pH of the duodenum (5.7 – 6.5) compared to bile (6.2 – 8.5) (Geigy, 1968a; Wilson, 2005). Lactobacilli, which are characterised by an innate acid tolerance (Dunne *et al.*, 2001) may outcompete other members of the biofilm communities at the distal liver end of the stent, which is in close

contact with the duodenum. Multivariate statistical analysis showed significant differences between microbial community structures of biliary and pancreatic stents. The different conditions prevailing in the pancreatic duct, arising from the secretion of the alkaline pancreatic juice (pH 7.5 – 8.8), which contains digestive enzymes including trypsinogen, chymotrypsinogen, carboxypeptidase, pancreatic lipase and amylase (Geigy, 1968b), provide a likely explanation for such differences. However, the group of biofilms sampled from pancreatic stents was characterised by a high within group heterogeneity, thus making general theories inconclusive until further investigations are made.

4.1.4 Detecting bacterial species that were active within the biofilm

As the rRNA content of a cell is positively correlated with its growth rate (Schaechter *et al.*, 1958) and metabolically active cells were shown to contain more rRNA than resting or dormant cells (Lee & Kemp, 1994; Poulsen *et al.*, 1993) a comparison of 16S rRNA and 16S rDNA SSCP fingerprints is supposed to give indications on metabolically active and inactive bacteria. *Bacteroides* sp., *Enterococcus faecalis* and *Lactobacillus* sp. were both present and active at the time of sampling in selected stents (by observing major bands on both the 16S rRNA and the 16S rDNA SSCP fingerprints). Also, several minor bands were detected on the DNA fingerprints but absent on the RNA fingerprints. The most prominent among these in the examined biofilms was closely related to *Streptococcus anginosus*. This suggests that although present at the time of sampling, they were perhaps not active. On the other hand, *Prevotella nanceiensis* was only detected on the RNA fingerprint of one biofilm, and can thus be assumed to be active but at low abundance. However, considering the difficulties of extracting sufficient amounts of ribosomal RNA from biliary stent samples, 16S rDNA SSCP fingerprinting as the tool to analyse the community structure of biliary stent biofilms is regarded as the better method of choice.

4.2 The use of culture-independent methods

This work represents the first detailed culture-independent community analysis of biliary stent biofilms in which molecular methods were used to generate sequence data of abundant microorganisms. To date, characterisation of bacterial community structure

of stent biofilms were obtained through either cultivation methods (Di Rosa *et al.*, 1999; Dowidar *et al.*, 1991; Leung *et al.*, 2000b; Molinari *et al.*, 1996; Speer *et al.*, 1988b; Zhang *et al.*, 2003) or fluorescence in situ hybridisation (FISH) (Swidsinski *et al.*, 2005). FISH methods reveal only the coarse structure of communities through application of group specific and several genus specific probes, while cultivation methods result in biases towards easily cultivable microorganisms and are time-consuming, which severely restricts the number of samples that can be processed. For example, only up to 31 samples were analysed using culture-dependent methods (Speer *et al.*, 1988b), while 133 samples were analysed in this study using culture-independent methods.

4.2.1 The application of fingerprinting methods

In this study, the principal method applied to characterise microbial communities in biliary stents was single strand conformation polymorphism (SSCP) fingerprinting. Recently, Smalla *et al.* compared three different fingerprinting methods: denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) and SSCP, and showed that all three methods led to a similar classification of the soil samples tested (Smalla *et al.*, 2007). While these fingerprinting methods may not have the power to describe the full microbial diversity of complex habitats, they have the advantage of being able to analyse a high number of samples simultaneously, thus making such fingerprinting methods ideal tools to gain an overview on microbial community composition of biliary stent biofilms across different hospitals and patients. Although T-RFLP may have a greater resolution power, as it is not restricted by gel-to-gel variations (Nunan *et al.*, 2005), the major benefit of SSCP and DGGE fingerprinting are their abilities to characterise sequences of predominant bands following reamplification. This results in a detailed picture on community compositions rather than just a general overview on community differences without phylogenetic affiliations. The phylogenetic affiliation of bands has been successfully applied in several studies from different habitats (Eichler *et al.*, 2006; Fracchia *et al.*, 2006; Mohr & Tebbe, 2006).

Although the amplified fragments include the highly informative variable regions V4 and V5 of the 16S rRNA (Neefs *et al.*, 1993), which have been shown to be appropriate for SSCP fingerprinting (Schmalenberger *et al.*, 2001), the relatively short length of the fragments (approximately 370 bp without primer sequences) does not always allow for

a detailed phylogenetic classification. For example, some members of the species *Klebsiella pneumoniae* and *Enterobacter cloacae* subsp. *dissolvens* share the same Com fragment, thus the detected SSCP phylotype 3 did not allow a classification to the species level. Therefore, it cannot be excluded that single phylotypes represent several bacterial species and possibly not all community members have been separated. However, the degree of resolution obtained with the complete 16S rDNA is also restricted (Ludwig *et al.*, 1998) and cases have been reported where 16S rDNA based analyses do not correlate with the DNA reassociation values determined by DNA hybridisation which has been used as the criterion for definition of bacterial species (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). In these cases, it seems that the resolution of 16S rDNA based analyses is low due to the small number of substitution between compared genes. Thus, to obtain a higher resolution, phylogenetic relationships need to be analysed by protein-encoding genes, which have been reported to evolve much faster than rRNAs (Ochman & Wilson, 1987). Therefore, proteins like DNA gyrase and the sigma 70 factor, which are essential for cell survival in bacteria and for which horizontal transmission of encoding genes is assumed to be rare have been used for inferring intrageneric relationships (Yamamoto & Harayama, 1998), such as in Enterobacteriaceae (Dauga, 2002). However, even such analyses do not necessarily give indications on functions of organisms observed to be predominant in a given ecosystem, as genome sequence analyses have revealed significant amounts of genetic information of microorganisms to be located on mobile genetic elements and been recently obtained by horizontal gene transfer (Chain *et al.*, 2006). Thus, it is clear that community structure and functions can be analysed at very different levels of resolution. As described above, no culture-independent information on community diversity of biliary stent biofilms was available and therefore SSCP fingerprinting was performed. This method proved to be very useful for studying this habitat and to obtain an overview of a high number of biliary stent biofilm communities originating from different patients and hospitals.

The amount of data generated from multiple species prevalence across 133 samples required the use of robust statistical tools that provide sensitive and readily interpretable results that were able to disclose slight or hidden differences in community structure. These tools have been successfully applied to analyse diversity data at the scale of macro- (Guerra-Garcia *et al.*, 2006; Wilber *et al.*, 2007) and microorganisms (Nelson, 2007; Van der Gucht *et al.*, 2005). Such multivariate routines

(as used here and described in the methods chapter 2.7.8.5) provided a convincing visual representation of large data sets where maps were constructed by placing samples with similar attributes close together, identified whether there were statistical differences between defined groups of stents, identified groups of species that tend to coaggregate together across stents and identified those species that primarily provided discrimination between groups of biofilms. Such routines proved to be an invaluable tool.

4.2.2 Validation of the SSCP method through analysis of 16S rDNA clone libraries of selected biliary stent biofilm communities

Random sequencing of 16S rDNA clone libraries is a useful tool for detailed analysis of microbial communities. Targeted against the universal marker 16S rDNA, this method generates a high amount of sequence data and reveals the fine-scale structure of the analysed community. As this method is quite laborious it was only applied to a selection of two biliary stents to analyse the community structure in more detail and to validate the applicability of SSCP fingerprinting with these samples. However, evaluation of diversity data has to be taken with care. Besides a PCR bias and overestimation of diversity due to the presence of multiple but different 16S rDNA copies in single microorganisms, the PCR-mediated generation of chimeras has to be taken into consideration. Recently, numerous corrupt 16S rRNA gene sequences within the public databases (GenBank, EMBL, RDP) have been reported (Ashelford *et al.*, 2005; Hugenholtz & Huber, 2003). It was estimated that 5% of records are likely to have substantial anomalies (Ashelford *et al.*, 2005), and beside poor sequencing and errors during assembly, most anomalies (64%) have been chimeras, artificial sequences generated from two or more phylogenetically different DNA templates during PCR amplification. A more recent survey on large 16S rRNA gene libraries (Ashelford *et al.*, 2006) revealed an even higher average anomaly content per library of 9% with approximately 91% of those anomalies being due to chimeric sequences, although the formation of chimeras has been documented for two decades (Shuldiner *et al.*, 1989). In fact, 27% and 15% of clones from both libraries, respectively, could be identified as chimeras as revealed with the chimera detection programs Bellerophon and Mallard (Ashelford *et al.*, 2006; Huber *et al.*, 2004). A high frequency of chimera is correlated with several parameters of the PCR, namely a high number of PCR cycles,

high template concentrations and decreased elongation time (Qiu *et al.*, 2001). Moreover, PCR artefacts, including the formation of chimera, increase with species diversity in the community amplified (Qiu *et al.*, 2001). This is in good accordance with the higher occurrence of chimera in library I, which is characterised by a higher microbial diversity compared to library II (12 and 9 OTUs at a similarity level of ≥ 98.5%, respectively). The range of chimeric sequences observed seems to be reasonable in comparison to 32% chimeric sequences having been observed after a 30 cycle PCR amplification of a defined mixture of 7 Actinobacteria (Wang & Wang, 1997).

Furthermore, the ecological interpretation of microheterogeneity observed in clone libraries has to be performed with care. During amplification of a defined mixture of 7 closely related environmental 16S rDNA sequences, the formation of at least 14% of new sequences containing aberrations was observed (Speksnijder *et al.*, 2001), thus not all sequence heterogeneities are due to real diversity within a microbial community. In addition to this artificially increased diversity, the interoperonic differences between rRNA operons can contribute to microheterogeneity observed in 16S rDNA clone sequences, as discussed in chapter 4.2.3. However, the cut-off level chosen for grouping of OTUs at ≥ 98.5% sequence similarity ensures an accurate classification of sequences to their respective OTU and should not be biased by the inherent microheterogeneity.

The heterogeneity between two microbial communities from different patients and hospitals observed during random sequencing of 16S rDNA clone libraries matches with the observations made during SSCP fingerprinting of a range of stents from different patients and hospitals, where a wide variety of different microbial communities were observed. Thus, the two stents analysed in detail by 16S rDNA clone library sequencing cannot be representative for all biliary stent microbial communities. However, they can be used as validation for the method chosen for biliary stent community analysis, the SSCP fingerprinting method. The comparison of microbial community structure as determined by SSCP fingerprinting and random sequencing of 16S rDNA clone libraries showed that sequences observed during SSCP fingerprinting covered a broad range of the cloned sequences. Furthermore, microorganisms indicated to be predominant by SSCP fingerprinting were also found to be predominant by analysis of clone libraries. In conclusion, the analysis of two selected microbial

communities of biliary stents showed the SSCP fingerprinting method to be a reliable and useful tool for the characterisation of biliary stent biofilm communities.

Direct culturing from stent I led, among microorganisms representative of the microbial community as determined with culture-independent methods, to the isolation of microorganisms not predominant in the biofilm such as *Propionibacterium* sp. or *Serratia marcescens.* This indicates an enrichment of minor community members during cultivation and confirms the bias, which is implied in culturing studies, thus underlining the importance of a thorough analysis of biliary stent biofilm community structure using appropriate culture-independent methods.

4.2.3 rRNA operon copy number and microdiversity

It has been reported that 16S rRNA gene sequences can differ up to several percent between multiple rRNA operons of a single strain (Mylvaganam & Dennis, 1992). Such sequence heterogeneity within single genomes can create a significant problem for culture-independent analysis of microbial communities since it can result in an overestimation of microbial diversity based on 16S rRNA approaches (Crosby & Criddle, 2003). This has led many authors to omit small-scale sequence differences encountered in environmental 16S rRNA gene clone libraries from estimates of diversity (Curtis *et al.*, 2002; Torsvik *et al.*, 2002). However, a careful analysis of 380 operons in 76 bacterial genomes (Acinas *et al.*, 2004) revealed divergence among operons to be small, with the vast majority of 16S rDNA sequences showing <1% nucleotide divergence and a high level of divergence was only observed among thermophilic bacteria, suggesting a much higher incidence of horizontal gene transfer in these bacteria than in other groups.

On SSCP fingerprints of biliary stent biofilm communities several SSCP phylotypes were characterised by several bands occurring concurrently. However, most of these showed an identical sequence (e.g. SSCP phylotypes *Bifidobacterium* sp. (26), *Fusobacterium* sp. (48, 54, 59), *Clostridium perfringens* (15) and *Bacteroides fragilis* (9)) and thus are most probably due to multiple conformations of the same single-stranded DNA molecule (Nakabayashi & Nishigaki, 1996; Schmalenberger *et al.*, 2001; Schwieger & Tebbe, 1998). Some SSCP phylotypes, however, showed sequence

heterogeneity between the different bands (e.g. SSCP phylotypes *Veillonella* sp. (41) and *Bacteroides ovatus* (12)), which did not exceed 1%.

For some of the microorganisms observed during analysis of 16S rDNA clone libraries of biliary stent biofilm communities, divergences in RNA operons have been described. *Enterococcus faecalis* is reported to contain 4 operon copies, of which one is divergent by 0.07% (1 nucleotide over the whole length of the 16S rRNA) (Acinas *et al.*, 2004) and *Escherichia* sp. and *Shigella* sp. are reported to contain 7 copies, with 3-6 different copies characterised by a divergence of up to 1.36% (21 nucleotides) (Acinas *et al.*, 2004). For Streptococci, 4 – 7 operon copy numbers are described. Whereas in some strains all copies are identical, in others up to 3 different copies with a divergence of up to 0.19% (3 nucleotides) are reported (Acinas *et al.*, 2004). For *Veillonella* sp. 4 different operon copies have been described, which are divergent up to 1.43% (22 nucleotides) (Marchandin *et al.*, 2003). Thus, the cut-off level chosen for grouping of OTUs at ≥ 98.5% sequence similarity ensures an accurate classification of sequences to their respective OTU and should not be biased by the inherent microheterogeneity.

The copy number of 16S rRNA genes can vary from 1 to 15 among eubacterial genomes (Klappenbach *et al.*, 2001), thus may lead to an overestimation of abundance for species characterised by a high copy number. This effect was also observed in the metagenome shotgun sequencing project of the Sargasso Sea (Venter *et al.*, 2004), where multiple phylogenetic markers were used to analyse phylogenetic diversity. The gamma-Proteobacteria, which frequently have more than 5 rRNA operon copies, were estimated to be higher abundant when using rRNA genes in comparison with other phylogenetic markers, like heat shock protein 70 or elongation factors EF-Tu and EF-G, which are involved in the elongation process during the translation of proteins. The greatest number of rRNA operons per genome known, can be found among spore-forming bacteria and *Bacillus subtilis* (Loughney *et al.*, 1983) and *Clostridium paradoxum* (Rainey *et al.*, 1996), possess 10 and 15 copies, respectively. Several hypotheses have been proposed to explain the wide variation observed in rRNA operon copy number. It is generally assumed that multiple copies of rRNA operons in prokaryotic organisms are required to achieve high growth rates. However, recent analysis indicated that the number of rRNA genes correlates with the rate at which bacteria respond to resource availability (Klappenbach *et al.*, 2000). Interestingly, phylogenetic groups observed as abundant in gastrointestinal ecosystems are

characterised by a relatively high number of rRNA operons. Members of the genus *Veillonella*, described to be of high prevalence in biliary stent biofilms are reported to contain 4 copies (Marchandin *et al.*, 2003), *Bacteroides* between 4 and 7 (Klappenbach *et al.*, 2000; Klappenbach *et al.*, 2001), Lactobacilli 4 – 6 (de Vries *et al.*, 2006; Fogel *et al.*, 1999), Bifidobacteria 2 -5 (Candela *et al.*, 2004), Streptococci 4 – 7 (Acinas *et al.*, 2004), *Fusobacterium nucleatum* 5 (Acinas *et al.*, 2004), *Enterococcus faecalis* 4 (Acinas *et al.*, 2004) and *Escherichia coli* 7 (Klappenbach *et al.*, 2000). Thus, all phylotypes observed to be abundant in biliary stent biofilms are reported to contain between 4 – 7 rDNA copies per genome, such that their sensitivity of detection by SSCP fingerprinting or random sequencing of 16S rDNA libraries is highly similar. Exceptions to this are, as shown above, Bifidobacteria, which due to their lower copy number are maybe slightly underrepresented and *Clostridium perfringens*, which was only observed in 15% of all stents, despite the fact that *C. perfringens* was reported to contain 10 16S rDNA gene copies (Acinas *et al.*, 2004).

4.3 Ecological significance of bacterial species observed in biliary stents

The major groups of bacteria observed in biliary stent biofilms and their ecological and metabolic features are discussed in this chapter. The most abundant phylotype observed during SSCP fingerprinting was *Veillonella* sp., small, non-motile, non-fermentive, strictly anaerobic, gram-negative cocci, which are associated with the oral, intestinal, genitourinary and respiratory microbiota of humans and animals. In particular, *Veillonella* sp. are ubiquitous members of the tongue, dental plaque and the buccal mucosa (Hughes *et al.*, 1988). They constitute as much as 5% of the initial biomass of the dental plaque (Mager *et al.*, 2003), a multispecies biofilm with Streptococci as primary components. *Veillonella* sp. are not able to metabolise carbohydrates, but are dependent on the fermentation of organic acids to propionic and acetic acids, carbon dioxide and hydrogen. They are able to grow at low pH and are often associated with oral infections, bite wounds, head, neck and various soft tissue infections (Theron *et al.*, 2003). Recently, they have been implicated as pathogens in severe human infections (Bhatti & Frank, 2000; Liu *et al.*, 1998; Marchandin *et al.*, 2001; Theron *et al.*, 2003).

Studies on *Veillonella* have largely been performed on isolates obtained from the oral cavity. In order to gain an insight into the biofilm formation, Hughes *et al.* (Hughes *et al.*, 1988) analysed *Veillonella* sp. isolated from tongue, dental plaque and buccal mucosa for their coaggregation properties and showed that tongue isolates coaggregated with strains of *Streptococcus salivarius*, a predominant inhabitant of the tongue surface, whereas subgingival dental plaque isolates coaggregated with bacteria typically present in this environment, but not with *S. salivarius*. Accordingly, the authors postulated that *Veillonella* species are distributed on oral surfaces that are occupied by their coaggregation partners, whereas the majority of *Veillonella* sp. isolated from sugingival plaque displayed coaggregation with multiple streptococcal strains (Hughes *et al.*, 1988). Coaggregation as an important factor for *Veillonella* infection was further supported by studies with gnotobiotic rats, where *Veillonella* sp. alone proved to be unable to colonise teeth surfaces. However, when *Veillonella* sp. were administered following a monoinfection with *Streptococcus mutans*, cell numbers of *Veillonella* sp. on teeth surfaces increased by 1000 fold when a coaggregating instead of a non-coaggregating *Veillonella* sp. strain was used (McBride & Van der Hoeven, 1981). In a recent study assessing 12 *Veillonella* sp. isolates of the oral cavity and the intestine for their coaggregation properties with 6 streptococcal strains, 7 of the 12 *Veillonella* sp. showed promiscuous and strong coaggregation (Palmer *et al.*, 2006). In particular, isolates loosely related to *V. parvula* and *V. dispar* exhibited strong coaggregation properties. Thus, in biliary stent biofilms *Veillonella* sp. may rely on their coaggregation properties to attach more easily to the biofilm. In fact, a strong correlation between the presence of SSCP phylotype 41 (closely related to *V. parvula* and *V. dispar*) and the presence of SSCP phylotype 35 (closely related to *Streptococcus anginosus*) was observed (see figure 4.1), which could hint at a possible coaggregation between those partners.

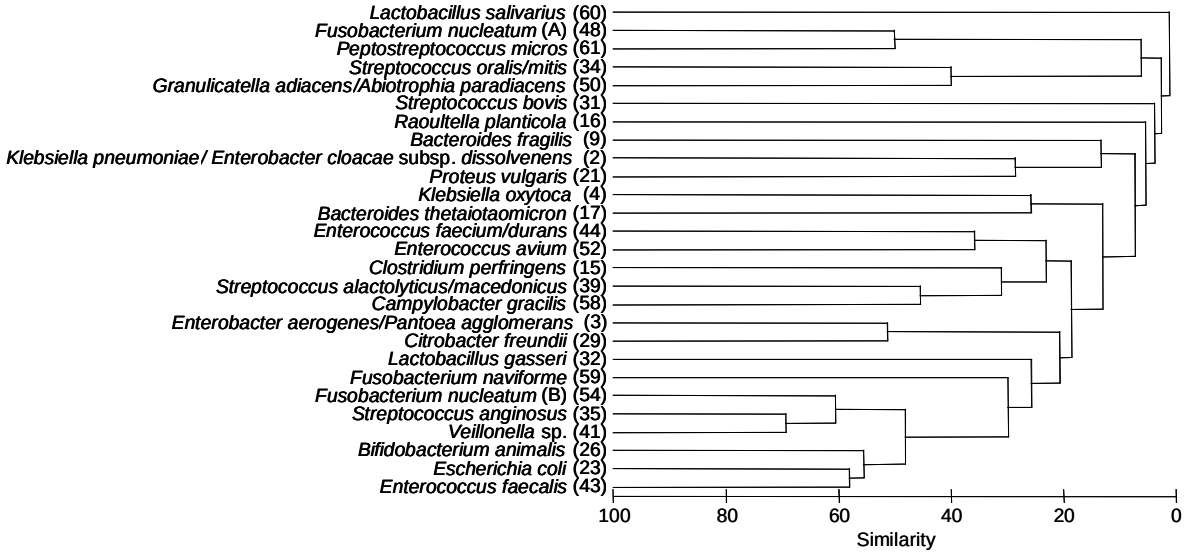

Figure 4.1: Hierarchical cluster dendrogram based on Bray-Curtis similarity from presence/absence data of SSCP phylotypes that were present within more than 1 patient from both BS and SZ hospitals. The x-axis indicates the % similarity in distribution/aggregation patterns that species share within stent biofilms. The number in parentheses following the species name indicates its SSCP phylotype number.

Viridans streptococci (VS) were the major facultative anaerobic, non-sporing, gram-positive streptococci observed in biliary stent biofilms. VS can be isolated as part of the normal microbiota of the respiratory, genital and alimentary tracts and are particularly abundant in the oral cavity. However, some species of VS are the most common etiologic agents of subacute infective endocarditis and can cause a variety of pyogenic infections (Facklam, 2002). Species of VS are also playing an increasing role in infections among immunocompromised patients (Shenep, 2000). On the basis of the 16S rRNA gene sequences, species of VS are divided into five major groups, the mutans, salivarius, sanguinis, mitis and the anginosus group (Facklam, 2002). Sequences highly related to *S. anginosus* (SSCP phylotype 35) were frequently observed in biliary stent biofilms. In Taiwan, it was found that VS accounted for 9% of all cases of culture-proven bacterial meningitis in adults and that species of the anginosus group comprised more than 80% of isolates (Chang *et al.*, 2002). Sasaki *et al.* (Sasaki *et al.*, 1998) reported the frequent PCR-based detection of *S. anginosus* in samples from surgical specimens of most oesophageal and some gastric cancers, whereas, it was rarely found in matched non-cancerous tissues. Thus, it has been postulated that *S. anginosus* may have a significant role in the carcinogenic process of human cancers (Shiga *et al.*, 2001; Tateda *et al.*, 2000). A more recent subject based

study revealed genotypes of *S. anginosus* isolated from cancer tissue being identical to those from the dental plaque of the same patients, indicating that dental plaque could be the dominant reservoir for the spread of *S. anginosus* to cancer tissue. Whether a similar route is taken for establishment of *S. anginosus* in biliary stents remains to be investigated.

Biofilm formation is thought to proceed through processes of adhesion and multiplication. The first step is the attachment of primary colonisers to the surface through specific or non-specific physico-chemical interactions. In dental plaques Streptococci are clearly the primary colonisers, as they constitute the majority of observed microorganisms in the initial microbiota of oral biofilms (Diaz *et al.*, 2006; Nyvad & Kilian, 1990). The initial attachment is facilitated by salivary components that act as receptors for bacterial adhesins (Scannapieco, 1994) and intrageneric coaggregation properties of oral streptococci (Kolenbrander *et al.*, 1990). Similar to the adhesins of Streptococci, which mediate the adherence to salivary proteins (Demuth *et al.*, 1996; Egland *et al.*, 2001; Takahashi *et al.*, 2002), adhesins to fibronectin and vitronectin known to coat biliary stent surfaces introduced in the human host (Yu *et al.*, 1996) may play a role in primary colonisation of biliary stents. Several gram-positive cocci, including *S. anginosus* (Willcox *et al.*, 1995) were shown to adhere to fibronectin (Joh *et al.*, 1999), thus suggesting a role of this mechanism in primary biliary stent colonisation. Multiplication and microcolony formation of the primary colonisers leads to the development of a young biofilm to which then secondary colonisers such as *Veillonella* can attach by adherence to the bacterial cells already present, thus leading to the development of a multispecies biofilm (Rickard *et al.*, 2003).

Bifidobacteria are non-sporing, non-motile, anaerobic, gram-positive rods and while more typically found in the colon, they are also found in the upper gastrointestinal tract or the vagina. They can hydrolyse a wide range of polysaccharides, which are non-digestible for humans and are widely used as probiotics in the food industry (Marco *et al.*, 2006). Furthermore, they provide the human host with a number of vitamins. They are acid-tolerant and only very rarely involved in human infections. Bifidobacteria were more frequently observed in stents from SZ compared to stents from BS, and sequences obtained were related to *B. animalis* which have been extensively used in functional dairy products (Masco *et al.*, 2005), rather than *B. longum, bifidum, adolescentis* and *angulatum* which are assumed to be the predominant species in the

human gastrointestinal tract (Gueimonde *et al.*, 2004; Hopkins & Macfarlane, 2002; Mangin *et al.*, 2006; Nielsen *et al.*, 2003; Satokari *et al.*, 2001). In general, bifidobacterial strains that are associated with the gastrointestinal tract harbour a bile salt hydrolase (*bsh*) (Begley *et al.*, 2006). *Bsh* has also been observed in *B. animalis* and a recent study reported evidence of the *in vivo* function of bsh of a *B. animalis* strain during its transit in the intestine of pigs (Lepercq *et al.*, 2004). It may be that such activity is also expressed by *B. animalis* strains in biliary stent biofilms. In addition to a high bsh activity (Begley *et al.*, 2006), Bifidobacteria also display other mechanisms to counteract bile toxicity, such as active extrusion of bile salts. The respective transporter has been recently identified in *B. longum* and was shown to simultaneously confer resistance to chloramphenicol and erythromycin (Price *et al.*, 2006). Moreover, bile acids affect the lipid composition of Bifidobacteria (Gomez Zavaglia *et al.*, 2002; Ruiz *et al.*, 2007). As bile salts dissipate membrane potential, rendering the bacterial membrane permeable to protons and causing cell death (Kurdi *et al.*, 2006), such adaptational changes enable the bacteria to survive under bile challenge. The adaptation to bile of *Bifidobacterium* is the result of a sum of complex phenomena directed to maintain the optimal viability of the cell under stress conditions, including a higher tolerance to low pH and induced expression of glycoside-hydrolysing activities for the assimilation of non-digestible carbohydrates (Noriega *et al.*, 2004; Sanchez *et al.*, 2005).

Fusobacteria are non-motile, non-sporing, gram-negative, anaerobic bacteria acquiring the name from their “fusiform” morphology. They are abundant colonisers of the oral cavity and the intestine and ferment peptides and amino acids, but are not able to use sugars. *Fusobacterium nucleatum* plays an important role in the progression of periodontal disease and has been associated with many polymicrobial anaerobic infections (Zilm & Rogers, 2007) and could be isolated from skin ulcers, septic arthritis or endocarditis (Bolstad *et al.*, 1996). In the initial stages of the periodontal disease process, *Streptococcus* sp. and other bacteria adhere and colonise, which sets the stage for *F. nucleatum* to coaggregate with early colonisers and permit late colonisers to form a biofilm (Bolstad *et al.*, 1996). Hence, they have a special role as bridging organisms (Kolenbrander *et al.*, 2002). In addition to their ability to coaggregate with many different bacteria, *F. nucleatum* has been described to promote physico-chemical changes such as alkalisation (Bickel & Cimasoni, 1985; Diaz *et al.*, 2002), which could

allow successors to colonise and proliferate. Whether *F. nucleatum* plays a similar role in biliary stent biofilms remains to be elucidated.

Enterococci are facultative anaerobic, non-sporing, gram-positive cocci. In particular, the two species *E. faecalis* and *E. faecium* are regular members of the intestinal microbiota. They can survive under very harsh conditions and are resistant to bile salts and exhibit a native tolerance to the bactericidal activities of penicillin and other cell wall-active antibiotics (Fontana *et al.*, 1990). *E. faecalis* is frequently found to be the causative agent of urinary tract, abdominal and wound infections as well as bacteraemia and infective endocarditis (Moellering, 1992) and is particularly problematic in hospitalised patients. The majority of clinical isolates of *E. faecalis* have the ability to form a single species biofilm *in vitro* (Mohamed *et al.*, 2004; Toledo-Arana *et al.*, 2001). Although the genetic basis of biofilm formation by *E. faecalis* is largely unknown, several factors that promote biofilm formation have been identified (van Merode *et al.*, 2006a). *E. faecalis* possesses several adhesins, which promote aggregation and adhesion. One of these proteins frequently detected in Enterococci causing infections and therefore assumed to be associated with adhesion and infection is termed aggregation substance (Agg). In fact, cells expressing Agg show an increased adherence to immobilised fibronectin and vitronectin (Rozdzinski *et al.*, 2001). The second surface protein, enterococcal surface protein Esp has been shown to be associated with the capacity of *E. faecalis* to form a biofilm on a polystyrene surface (Toledo-Arana *et al.*, 2001). Both these proteins have been thus suggested to be associated with the ability of the microorganism to adhere to and subsequently infect host tissue, or to spread within hospital settings. However, not only surface proteins, but also the surface hydrophobicities and charges of both the substratum and the adhering microorganism significantly influence adhesion. In fact, heterogeneity of *E. faecalis* cultures regarding their surface charge was recently observed to be important for adhesion and biofilm formation of *E. faecalis*, and the majority of biliary stent isolates recovered by van Merode *et al.* (van Merode *et al.*, 2006b) showed such heterogeneity, indicating that such isolates have a significant advantage in colonising surfaces. As *E. faecalis* has been reported as an important member of biliary stent biofilms, prophylactic antibiotic treatment has been suggested (Leung *et al.*, 2000a; Rerknimitr *et al.*, 2002). However, Enterococci are known for their acquired multidrug-resistance through plasmids or conjugative transposons (Amyes, 2007) and the emergence of vancomycin resistant enterococci is posing severe problems. Respective

multidrug-resistant strains are practically resistant to all antibiotics of proven efficacy (Amyes, 2007; Bonten *et al.*, 2001). The analysis of 21 enterococcal strains isolated from biliary stents for their antibiotic resistance pattern revealed that while no vancomycin resistant strains were detected, 5 out of 13 *E. faecalis* strains and 1 out of 8 *E. faecium* strains were shown to be multidrug resistant (Donelli *et al.*, 2004). Nevertheless, the frequency of vancomycin resistant clones is increasing (Goossens *et al.*, 2003; Health Protection Agency, 2006) such that the appearance of respective antibiotic resistant clones in biliary stents seems to be just a matter of time.

The anaerobic *Bacteroides* sp. are non-motile, non-sporing, gram-negative rods and can use a wide range of carbohydrates and polysaccharides. They are among the most predominant genera of intestinal microbiota (Hold *et al.*, 2002) and have been found to be dominant among bacteria on the ileal mucosal surface (Wang *et al.*, 2005). *B. thetaiotaomicron* can break down a broad array of dietary polysaccharides *in vitro* (Xu & Gordon, 2003). Analysis of its genome showed that it has, among sequenced genomes, the largest repertoire of genes involved in acquisition and metabolism of polysaccharides, including 226 predicted glycoside hydrolases and 15 polysaccharide lyases (Sonnenburg *et al.*, 2005). Analysis of other *Bacteroides* sp. such as *B. fragilis* (Kuwahara *et al.*, 2004) and *B. vulgatus* (Xu *et al.*, 2007) also revealed exceptional capabilities to degrade polysaccharides, however, to not such extent as indicated for *B. thetaiotaomicron*. In a study using germ-free mice, which were subsequently colonised with *B. thetaiotaomicron*, transcriptional profiling showed a selective induction of polysaccharide-binding proteins and glycoside hydrolases depending on the diet (Sonnenburg *et al.*, 2005). Epithelial surfaces in the human gastrointestinal tract are covered by a layer of mucus, which prevents most microorganisms reaching and persisting on the mucosal surface. Mucins are chemically and structurally diverse molecules, but they invariably contain large quantities of galactose and hexosamines with lesser amounts of fucose. Strongly polar groups such as neuraminic (sialic) acids and sulphate are also present, although at a highly variable degree (Quigley & Kelly, 1995). Large complex polymers such as mucin need to be degraded by several different hydrolytic enzymes to smaller oligomers, monosaccharides and amino acids before they can be assimilated by intestinal microorganisms. So although the breakdown of mucin and other complex organic molecules is a cooperative activity in the gut microbiota, some microorganisms like Bacteroidetes species can produce several different glycosidases, which allows them to extensively digest mucin. Mucins

are secreted by the gallbladder epithelium, are the primary constituent of gallbladder mucus, and have been identified as constituent of biliary sludge (Zhang *et al.*, 2003). Thus, the capability to degrade these molecules could offer a selective advantage for growth in biliary stents. In fact, studies on *B. thetaiotaomicron* showed that it degraded mucus polysaccharides *in vivo* when other polysaccharides were absent from the diet (Sonnenburg *et al.*, 2005) and studies on mucin gels revealed that they are rapidly colonised especially by *Bacteroides* sp. (Macfarlane *et al.*, 2005). *B. fragilis* treated with bile salts showed an increased resistance to antimicrobial agents, an increased bacterial co-aggregation, adhesion to intestinal epithelial cells and biofilm formation (Pumbwe *et al.*, 2007). The high concentrations of bile salts in the stent environment thus may induce biofilm formation of *Bacteroides* sp. in biliary stents.

The non-sporing, facultative anaerobic, gram-negative Enterobacteriaceae are a large family including approximately 30 genera. Many of these are typical members of the gastrointestinal microbiota. Some of the Enterobacteriaceae are opportunistic pathogens and virulent strains of *Escherichia coli* are the major causative agent of urinary tract infections (Yamamoto, 2007). In general, Enterobacteriaceae are more resistant to bile than gram-positive bacteria, and bile salts are even used as supplement in selective enrichment media for these microorganisms (Begley *et al.*, 2005a). Salmonellae have the capability to colonise the gallbladder (Prouty *et al.*, 2002) and also *E. coli* is commonly isolated from the gallbladder and bile of animals and humans (Flores *et al.*, 2003). Salmonellae form biofilms on the surface of gallstones, and efficient biofilm formation was dependent on the presence of bile. It was thus hypothesized that Salmonellae have the ability to sense bile and the presence of bile leads to induction of bacterial factors that may promote biofilm formation. However, no further information on other Enterobacteriaceae and bile response is available.

The non-sporing, non-motile, gram-positive Lactobacilli are mostly microaerophilic and typical inhabitants of the reproductive and gastrointestinal system. They gain energy by fermentation of sugars and very seldom cause infections in humans. In general, Lactobacilli strains that are associated with the gastrointestinal tract harbour a bile salt hydrolase (*bsh*) (Begley *et al.*, 2006). They are routinely used as probiotics (Marco *et al.*, 2006). In this context *Lactobacillus* sp. are often screened for their bile tolerance and hydrolase activity and it could be shown in pigs (De Smet *et al.*, 1998) that bsh

activity was enhanced by oral administration of *Lactobacillus reuteri*. This caused a greater drain on the bile salt pool, resulting in a loss of feedback inhibition on bile salt synthesis and an increased conversion of cholesterol into bile salts. Because of this, the serum cholesterol levels in the pigs were lowered. A general observation is that bile tolerance is a strain-specific trait and cannot be generalised for all Lactobacilli (Chateau *et al.*, 1994; Jacobsen *et al.*, 1999). Moreover, it could be shown that bsh activity and resistance to toxicity of conjugated bile salts are unrelated properties in Lactobacilli (Moser & Savage, 2001).

The obligate anaerobic *Clostridium* sp. are spore-forming, gram-positive rods. However, even though great advances in improving the classification of bacteria have progressed over the past decades, especially with the use of molecular tools, knowledge regarding the natural interrelationships within the genus *Clostridium* is still fragmented. It was stated more than a decade ago by Collins *et al.* that “the genus *Clostridium* is clearly in need of major revision, and the rRNA structures defined in this and previous studies may provide a sound basis for future taxonomic restructuring” (Collins *et al.*, 1994). Collins *et al*. (Collins *et al.*, 1994) showed that a significant amount of the clostridial species, including *Clostridium butyricum*, the type species of the genus, belong to a well defined cluster (designated cluster I), whereas the remaining clostridial species exhibited very considerable degrees of phylogenetic diversity and formed numerous clusters and individual lines of descent. An overall of nineteen clusters have been defined and the respective cluster nomenclature is still in use (Spring *et al.*, 2003; Wang *et al.*, 2005), even though some new genera have been defined in the meantime, such as *Moorella* comprising the former *Clostridium thermoaceticum* or *Oxobacter* comprising the former *C. pfennigii* (Collins *et al.*, 1994). An overall of 4 phylotypes were observed in biliary stent biofilms, which cluster with sequences of members of the genus *Clostridium*. Phylotype 22 and 28, respectively, were observed in 2 stents each and showed high similarity to either uncultured bacterium SJTU F 01 96 (EF398274) or to uncultured bacterium J5 – 58 (DQ113770), and in a phylogenetic analysis belong to cluster XIVa (the so-called *C. coccoides* cluster which also comprise various *Eubacterium* and *Ruminococcus* strains) and cluster XI, respectively. Phylotype 8, observed in 1 stent showed high similarity to *C. ramosum*, a cluster XVIII strain, and phylotype 15, observed in 10 stents was related to *C. perfringens*, a cluster I *Clostridium* strain. However, analyses on the diversity of human intestinal microbiota have generally observed Cluster XIVa and Cluster IV (also

termed the *C. leptum* cluster) clostridia in high abundance in the human gastrointestinal tract (Eckburg *et al.*, 2005; Hold *et al.*, 2002; Zoetendal *et al.*, 2006) accounting for 28% and 25% of the faecal microbiota, respectively (Lay *et al.*, 2005). Cluster XIVa clostridia observed to be abundant on the ileal mucosa by Wang *et al.* (Wang *et al.*, 2005) were almost absent from stent biofilms. During the analysis of the gut contents, these groups were also absent from the upper gastrointestinal tract (Hayashi *et al.*, 2005). In contrast to above mentioned clostridial clusters, organisms related to *C. perfringens* were not abundant in the gastrointestinal tract. *C. perfringens* is one of the most pathogenic clostridia in humans and has been associated with several diseases, including gas gangrene (clostridial myonecrosis), necrotising enterocolitis, antibiotic-associated diarrhoea, and acute food poisoning (Fallani *et al.*, 2006). *C. perfringens* has also been isolated from human bile (Sakaguchi *et al.*, 1983), thus suggesting this organism to be capable to survive in this habitat.

Within the complex microbiota of the gastrointestinal system many metabolic interactions take place, which could also play a role in microbial communities of biliary stents. There are positive advantages of a community, such as degradation of complex macromolecular substrates like mucin, which is a task that only very few individual microbes can accomplish on their own, but the combined capabilities of several community members make this easy to achieve (Macfarlane *et al.*, 2005). Moreover, mixed biofilm populations proved to be more efficient in digesting polysaccharides, than non-adhering communities (Macfarlane & Macfarlane, 2006). Another example for a simple “food web” as it has been studied in oral cavity biofilms is the metabolic cooperation of Streptococci and Veillonellae. Streptococci catabolise carbohydrates to short-chain organic acids, on which Veillonellae rely, since they are not able themselves to catabolise carbohydrates (Palmer *et al.*, 2006). Such metabolic interactions are also conceivable for biliary stent biofilm communities.

4.4 Metabolic transformations possibly present in biliary stent biofilms

Following the first steps of identifying the predominant microbial members of biliary stent biofilms, the metabolic reactions and transformations that occur in such biofilms are interesting to elucidate. In the current work, bacterial isolates and biofilm

community DNA were examined for their potential to carry out two bacterial modifications of bile acids, which are known to occur in the large bowel.

Bile salt hydrolase (*bsh*) activity is known to occur in a large number of predominantly gram-positive intestinal genera, but is usually absent from bacteria isolated from environments were bile salts are not present (Begley *et al.*, 2006). Possible benefits for having a *bsh* such as enhancing survival, detoxification or release of nutritional amino acids have been described in the introduction and these factors could also be beneficial in the environment of biliary stent biofilms. The investigation of different bacterial biliary stent isolates proved bsh activity to be present in 4 of the 5 enterococcal strains analysed. Thus, an expression of this activity in biliary stent biofilms is conceivable, although no proof of expression *in vivo* was made. Bsh activity was absent from two *Streptococcus* isolates and a *Pediococcus* isolate investigated, in accordance with the fact that bsh activity has never been reported from members of these genera. However, it is likely that Lactobacilli, Bifidobacteria and *Bacteroides* strains that were shown to be present in biliary stent biofilms using culture-independent methods in this study, but of which no biliary stent isolates were available, also harbour bsh activity since bsh genes have been described to be widespread among members of these genera (Begley *et al.*, 2006; Christiaens *et al.*, 1992; Kawamoto *et al.*, 1989; Kim *et al.*, 2005; Ridlon *et al.*, 2006). Among the Clostridia, so far only *C. perfringens* has been shown to harbour a bsh (Coleman & Hudson, 1995), which possibly equip this species with a selective advantage to colonise biliary stents. However, a BLAST search indicated genes encoding proteins with high similarity to bsh of *Enterococcus faecium* FAIR-E 345 (AY260046) also to be present in the genomes of the *Clostridium* cluster XIVa strains *Ruminococcus gnavus* ATCC29149 (AAYG02000005, 60% identity), *Ruminococcus obeum* ATCC29174 (AAYO02000021, 61% identity), *Dorea longicatena* DSM13814 (AAXB02000007, 61 % identity) and *Clostridium* sp. L2-50 (AAYW02000018), whereas it seems to be absent from cluster IV clostridia.

In contrast to the widespread *bsh* genes, the pathway for the 7α-dehydroxylation encoded by the bile acid inducible (*bai*) genes is only known from Clostridiales (Ridlon *et al.*, 2006). Initial reports on this activity identified it to be present in *C. bifermentans* (Hayakawa & Hattori, 1970), *C. sordellii* (Hayakawa & Hattori, 1970) and closely related organisms (Hirano *et al.*, 1981) of the cluster XI, but also to be present in *C. leptum* (cluster IV) (Doerner *et al.*, 1997). However, significantly higher activities were

observed in strains previously classified as Eubacteria (Doerner *et al.*, 1997; White *et al.*, 1988) and recently classified as *C. scindens* (Kitahara *et al.*, 2000) belonging, like most 7α-dehydroxylating bacteria, to cluster XIVa. Further highly active 7α-dehydroxylating bacteria were classified as *C. hiranonis* strains (Kitahara *et al.*, 2001) and belong to cluster XI. Thus, although several SSCP phylotypes of the genus *Clostridium* have been identified in biliary stent biofilm communities (SSCP phylotypes 8, 15, 22 and 28), these are not among the representatives for which a 7α-dehydroxylating activity has been described (Doerner *et al.*, 1997). Moreover, a PCR assay previously developed to detect bai genes, encoding enzymes involved in 7α-dehydroxylation of *Clostridium scindens* VPI 12708 and *Clostridium hiranonis* TO931 (Wells *et al.*, 2003) and validated to detect such genes in various *C. scindens* strains and in faecal DNA, failed to give an amplification product from biliary stent biofilm communities tested. This indicates that such genes are not abundant in biliary stent biofilm communities. However, since the primers applied have been reported to fail to give amplification products also from *C. leptum* (Wells *et al.*, 2003), they are not covering the whole diversity of genes coding for enzymes involved in 7α-dehydroxylation, and it thus cannot be finally excluded that such activities are present in the biofilms.

Deconjugation of taurine- or glycine-conjugated bile acids as catalysed by bsh may provide a means of obtaining cellular carbon, nitrogen and sulphur for some bacterial species. Glycine can be oxidised by various anaerobic bacteria catalysed via the glycine-cleavage system (Okamura-Ikeda *et al.*, 1993) to CO_2, NH_3, methylene-tetrahydrofolate and NADH. Alternatively, it can be reduced to acetyl phosphate by glycine reductases (Andreesen, 2004). Thus, glycine can act as oxidant and reductant. Taurine can be transformed by at least four enzymes (see (Cook & Denger, 2002) for review), but only two of them are reported to be induced by taurine. A taurine dehydrogenase releasing ammonium from taurine has so far only been reported from *Achromobacter xylosoxidans* (Kondo *et al.*, 1973), whereas taurine:pyruvate aminotransferase (*tpa*) has been found in many aerobes and anaerobes (Cook & Denger, 2002), including *Bilophila wadsworthia*, a typical member of the gut microbiota. The transaminase transfers the aminogroup into alanine, from where it can be released by alanine dehydrogenase, which also has been described from *B. wadsworthia* (Laue & Cook, 2000). Sufoacetaldehyde from taurine is transformed to sulphite and acetylphosphate (Cook & Denger, 2002) by a so-called acetyl-phosphate sulphite

acetyltransferase and sulphite can be used as electron acceptor and is reduced by a dissimilatory sulphite reductase to sulphide in *B. wadsworthia*. *B. wadsworthia*, which is a suspected pathogen and was recovered from different clinical specimens, especially appendicitis (Finegold *et al.*, 1992), was detected on SSCP fingerprints of two stents from one patient of SZ. Since it can be expected that taurine respiring organisms find an ecological niche in biliary stent biofilms, *B. wadsworthia* may be present in microbial communities of biliary stents in a regular manner, taking advantage of its metabolic properties, but in an abundance that is too low to be detectable on SSCP fingerprints. Thus, a specific PCR assay targeting the *tpa* gene of the degradative pathway for taurine (Laue *et al.*, 2006) was used to check for the presence of this metabolic trait. No positive PCR reaction was observed for biliary stent biofilm communities tested. Thus, the utilisation of taurine as electron acceptor by *B. wadsworthia* seems to be no universal characteristic of biliary stent biofilm communities.

4.5 Possible strategies to prevent biliary stent occlusion

The multifactorial process that leads to stent occlusion was already described in the introduction. In this work, it was shown that biliary stent biofilm communities do not constantly consist of the same major members but single biofilms are composed of several different bacterial species. In total, mostly members of the typical duodenal microbiota were observed in changing combinations. This indicates that primary colonisers responsible for the early attachment and microorganisms participating in sludge formation are interchangeable. Thus, a targeted antimicrobial therapy does not seem to be very promising since inhibited microorganisms are simply replaced by others. This is supported by a study of Leung *et al.* (Leung *et al.*, 2000b), in which all patients received antibiotic prophylaxis targeted against gram-negative aerobic bacteria, subsequently only gram-positive and gram-negative anaerobic bacteria were detected.

Another approach that hinders stent occlusion is the variation of employed stents in design and material and coating of stent material with antimicrobial or hydrophilic agents. Although the use of different materials and coatings appeared promising *in vitro*, efficiency in clinical trials was rarely observed (Catalano *et al.*, 2002; Schilling *et al.*, 2003; van Berkel *et al.*, 2004). One major factor in the clogging process seems to

be the occurrence of duodenal reflux, which introduces nutritional constituents and dietary fibres into the bile duct. The presence of dietary fibres in biliary stent biofilms could be also shown in this work, by analysis of selected samples with light and electron microscopy. The introduction of dietary fibres into the bile ducts and the inherent stents causes several problems. The mechanical obstruction of the stents contributes to the occlusion of the stent lumen and serves as a filter to retain precipitated substances and biofilm components. Moreover, the introduced nutritional constituents may serve as energy source for the biofilm communities, thus supporting further growth. Taking this into account, a reasonable approach for a new stent design would include some kind of mechanism that hinders at least partially the duodenal reflux, and thus introduction of bacteria and nutritional constituents into the bile ducts. In fact the development of antireflux biliary stents, which allow only one-directional flow of bile is underway and is being currently tested for its efficacy (Reddy *et al.*, 2006).

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Appendices

A1: Species richness table for stents from BS.

Biofilm community	Number of species detected
170205 L	7
170205 D	8
150305 L	7
150305 D	7
050705 L	4
050705 D	5
260805 L	9
260805 D	9
260905 L	3
260905 D	7
300905 L	7
300905 D	7
051005 L	7
051005 D	8
091105 EK L	8
091105 EK D	8
091105 CW L	6
091105 CW D	6
011205 L	6
011205 D	6
080206 L	5
080206 D	5
090206 L	5
090206 L ex	5
090206 D	7
030306 L	5
030306 D	6
080306 L	5
080306 D	7
130306 P	2
150306 BD L	2
150306 BD D	3
150306 DL L	6
150306 DL D	6
200306 L	7
200306 D	7
140606 L	3
140606 D	4
280606 L	4
280606 D	4
130706 L	6
130706 D	6

080806 L	1
080806 D	3
160806 L	3
160806 D	6
290806 KR L	5
290806 KR D	5
290806 KS L	6
290806 KS D	6
310806 L	4
310806 D	5
210906 L	9
210906 L ex	7
210906 D	10
051006 L	3
051006 D	3

A2: Species richness table for stents from SZ.

Biofilm community	Number of species detected
3006-0930 I L	10
3006-0930 I D	9
3006-0930 II L	9
3006-0930 II D	9
0507-1440 L	8
0507-1440 D	8
0607-1100 I P	4
0607-1100 I D	4
0607-1100 II L	2
0607-1100 II D	3
0807-1150 L	7
0807-1150 D	6
1107-0935 L	3
1107-0935 D	7
1207-1400 I L	7
1207-1400 I D	8
1207-1400 II L	6
1207-1400 II D	5
1807-1030 P	7
1807-1030 D	5
2107-1510 P	3
2107-1510 D	7
0308-1000 I L	6
0308-1000 I D	5
0308-1000 II L	7
0308-1000 II D	10
2309-1015 I L	10
2309-1015 I D	11
2309-1015 II L	8
2309-1015 II D	8

2309-1015 III L	11
2309-1015 III D	7
0510-1000 I P	8
0510-1000 I D	8
0510-1000 II L	7
0510-1000 II L ex	10
0510-1000 II D	6
0510-1000 III L	8
0510-1000 III L ex	9
0510-1000 III D	6
0111-1020 I L	7
0111-1020 I D	9
0111-1020 II L	3
0111-1020 II D	5
0711-1020 L	4
0711-1020 D	6
1011-1045 L	7
1011-1045 D	6
1511-1215 I L	7
1511-1215 I D	8
1511-1215 II L	9
1511-1215 II D	7
1711-1045 L	7
1711-1045 D	6
2811-1120 I L	5
2811-1120 I D	4
2811-1120 II L	6
2811-1120 II D	7
2811-1120 III L	7
2811-1120 III D	6
0612-1215 L	4
0612-1215 D	3
0712-1030 L	5
0712-1030 L ex	5
0712-1030 D	6
0201-1025 L	6
0201-1025 D	4
0903-0845 L	5
0903-0845 L ex	4
0903-0845 D	4
3003-0800 I L	5
3003-0800 I D	3
3003-0800 II L	5
3003-0800 II D	4
3003-0800 III L	5
3003-0800 III D	3

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